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I DECLARE THAT I PREPARE THIS TRANSLATION AND IT IS TRUE ENGLISH TRANSLATION MADE FROM KOREAN PATENT APPLICATION NO. 10-2003-0076629.

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【 Abstract Sheet 】

【 Abstract 】

The present invention relates to an EC SOD, a cell-transducing EC SOD fusion protein, and the composition containing the protein as an effective component. More particularly, the present invention relates to an EC SOD(extracellular superoxide dismutase) and an EC SOD fusion protein having enhanced Cell-transduction ability, and the composition for preventing or treating ROS (Reactive oxygen species) related diseases containing the protein as an effective component.

An EC SOD of the present invention has the effect of reducing ROS in skin cell, particularly, Cell-transducing EC SOD fusion protein is excellent in cell transduction, and ROS reduction in a cell, therefore, it may be useful in preventing and treating ROS related disease.

【 Representative Figure 】

FIG 11

【Key Word】

EC SOD, ROS (Reactive oxygen species), Cell-transduction ability, fusion protein

【Specification】

【Title of Invention】

EC SOD CELL-TRANSDUCING EC SOD FUSION PROTEIN AND COMPOSITION CONTAINING THE PROTEIN AS AN EFFECTIVE COMPONENT

【 Description of Drawings】

FIG. 1 is a photograph showing the results of immunohistochemical examination for the distribution pattern of EC SOD in mouse skin tissue. (A: a photograph showing the result of immunostaining with a rabbit anti-mouse EC SOD antibody. B: a photograph showing the result of immunostaining with preimmune rabbit serum as a negative control group.)

FIG. 2 shows the results of Northern blot analysis for the expression pattern of EC SOD in the epidermal and dermal layers of the mouse skin. GAPDH was used as a loading control.(**: significant difference at $p<0.05$.)

FIG. 3 shows the results of Northern blot analysis for the expression pattern of EC SOD in mice at different time points after UVA irradiation. GAPDH was used a loading control.(A: 5 kJ/m^2 of irradiation B: 25 kJ/m^2 of irradiation)

FIG. 4 shows the results of Northern blot analysis for the expression of EC SOD in mice at different time points after UVB irradiation. GAPDH was used as a loading control.(A: 2kJ/m^2 of irradiation B: 8kJ/m^2 of irradiation C: 15kJ/m^2 of irradiation)

FIG. 5 shows the results of Northern blot analysis for the expression of EC SOD in mice at different time points after the mice are treated with 8-MOP and, after one hour, irradiated with UVA. GAPDH was used as a loading control.(A: 5kJ/m^2 of irradiation B: 25kJ/m^2 of irradiation)

FIG. 6 shows the results of Northern blot analysis for the expression pattern of EC SOD (A) at different time points after removing tetracycline from a Tet off-MEF/3T3 inducible gene expression system (TET OFF) to induce the overexpression of EC SOD, as well as the activity of EC SOD (B).(TET ON: the case of presence of tetracycline TET OFF: the case of removal of tetracycline)

FIG. 7A is a graph showing, as a value relative to that of a control group, the amount of intracellular reactive oxygen species in a mouse EC SOD-overexpressed cell line at different time points after irradiation with 10 J/cm^2 of UVA. (TET ON: the case of presence of tetracycline TET OFF: the case of removal of tetracycline)

FIG. 7B is a graph showing, as a value relative to

that of a control group, the amount of intracellular reactive oxygen species in a mouse EC SOD-overexpressed cell line at different time points after irradiation with 20 mJ/cm^2 of UVB. (TET ON: the case of presence of tetracycline TET OFF: the case of removal of tetracycline)

FIG. 7C is a graph showing, as a value relative to that of a control group, the amount of intracellular active oxygen in a mouse EC SOD-overexpressed cell line at different time points after the cell line is treated with 0.1% 8-MOP and, after 30 minutes, irradiated with 2 J/cm^2 of UVA. (TET ON: the case of presence of tetracycline TET OFF: the case of removal of tetracycline)

FIG. 8 shows a restriction map of a human EC SOD overexpression vector according to the present invention.

FIG. 9 shows the result of Western blot analysis for EC SOD expressed in EC SOD-overexpressed HaCaT cells.

FIG. 10 shows the results of flow cytometric analysis for the ratio of cell death resulted from UV irradiation on EC SOD-overexpressed cells.

FIG. 11 shows a restriction map of a cell-transducing SOD fusion protein expression vector according to the present invention.

FIG. 12 is a photograph showing the results of electrophoresis analysis for a purified TAT-EC SOD fusion

protein and TAT-ΔHD/EC SOD fusion protein.

FIG. 13 is a photograph showing the results of electrophoresis for a purified TAT-EC SOD fusion protein, K10-EC SOD fusion protein and R9-EC SOD fusion protein.

FIG. 14 is a photograph showing the results of immunocytochemical observation indicating that a TAT-EC SOD fusion protein and TAT-ΔHD/EC SOD fusion protein of the present invention transduce into human keratinocyte cells so that they are located within the cell nuclei.(red: cell nuclei, green: fusion protein)

FIG. 15 shows the results of Western blot analysis for the cell transduction efficiencies of a TAT-EC SOD fusion protein and TAT-ΔHD/EC SOD fusion protein of the present invention.

【 Disclosure】

【Object of the invention】

【Technical Field and Background Art】

The present invention relates to an EC SOD, a cell-transducing EC SOD fusion protein, and the composition containing the protein as an effective component. More particularly, the present invention relates to an EC SOD(extracellular superoxide dismutase) and an EC SOD

fusion protein having enhanced Cell-transduction ability, and the composition for preventing or treating ROS (Reactive oxygen species) related diseases containing the protein as an effective component.

Reactive oxygen species(ROS) is naturally generated through metabolic process of a cell in every organism which gain energy by using oxigen. However, when they exposed to ROS excessively, bio-macromolecules such as protein in the cell, nucleotic acid and lipid may be damaged. These may cause cancer, stroke, arthritis, arteriosclerosis, and many kinds of inflamation, consequantly they may cause aging(Floyd, R. A., *FASEB J.*, 4, 2587-2597, 1990; Anderson, W. F., *Nature*, 392, 25-30, 1998; Halliwell B. et al., *Free radicals in biology and medicine*, Oxford University Press, Oxford).

In the prsent days, exposure to UV caused by external factors such as serious air pollution and disruption of the ozone layer becomes main reason for ROS genesis. ROS which is excessively generated by the above mentioned reasons brings damages in the skin and induces skin deseases such as skin aging and skin cancer.

In the human body, there are SOD(superoxide

dismutase), catalase, peroxidase as a deffensive means against ROS. Among them, SOD is a representative anti-oxidant enzyme. It has functions of removing reactive oxygen and protecting cells. SOD is classified into Cu/Zn SOD containing copper and zinc atoms, Mn SOD containing manganese atom, and extracellular superoxide dismutase (EC SOD) located in the cell surface or the extracellular fluid.

Among them, EC SOD contains copper and zinc atoms as in Cu/Zn SOD, but is characterized in that a heparin-binding domain is present in the C-terminal end. Since EC SOD has the heparin-binding domain, it is assumed that EC SOD will function to protect cell membranes by binding to the cell membranes. According to literatures, it was known that EC SOD plays a role in the body's defense mechanism in serums and extracellular matrices (Marklund et al, *Biochem. J.* 266, 213-219, 1990; Su et al., *Am J Respir Cell Mol Biol.*, Feb 16(2), 162-70, 1997; Luoma et al., *Thromb. Vasc. Bio.* 18, 157-167, 1998). Other literatures reported that gene therapy with EC SOD improves aorta restenosis in rabbits and alleviates collagen-induced arthritis in mice (Laukkanen MO et al., *Circulation*, 106, 1999-2003, 2002; Iyama S et al., *Arthritis & Rheumatism*, 44, 9, 2160-2167, 2001). Recently, it was reported that EC SOD could inhibit telomere shortening, a cell aging phenomenon in human

fibroblasts and extends the replicative life span of human fibroblast (Serra V et al., *J. Biol. Chem.*, 278, 9, 6824-6830, 2003). In addition, it was reported that the heparin-binding domain of EC SOD acts as a nuclear localization signal so that it is located within the nuclei of thymuses and testis cells so as to protect genomic DNA from oxidative stress and to regulate the DNA transcription sensitive to oxidation-reduction reaction (Ookawara T et al., *BBRC*, 296, 54-61, 2002). However, the distribution pattern of EC SOD in the skin of EC SOD is not yet known, nothing but it is reported that its activation is regulated by various cytokines in the skin fibroblast cells. (Marklund, *The journal of biochemisty* , 267, 10, Issue of April 5, 6696-6701, 1992)

Meanwhile, as the fact that certain proteins can effectively enter cells through cellular membranes is found, studies to use such proteins as transport means to transduce useful substances into cells are now actively performed. Typical examples of such proteins include HIV Tat protein, ANTP, VP22 protein, PEP-1 peptide, and the like (Lindgren et al., *TIPS* 21:99, 2001; Green et al., *Cell*, 55, 1179-1188, 1988). It is known that the cell-transduction ability of such proteins is caused by the

properties of a protein transduction domain (PTD) with the activity capable of crossing a cell membrane phospholipid bilayer (Fankel A. D. et al., *Cell*, 55, 1189-1193, 1988; Green M. et al., *Cell*, 55, 1179-1188, 1988).

Meanwhile, in case of drugs and proteins which are used as therapeutic agents cannot spontaneously cross the cell membrane in most cases. Resently, protein transporting region is disclosed as above mentioned, thereby promoting studies to introducing useful proteins to a cell by using thereof.

However, since it is not clear that how the above mentioned protain transporting domain was transported into a cell so far, and nor all kinds of proteins was transported by protain transporting domain.

【Technical Problem】

During proceeding studies on the characteristics of EC SOD, the present inventors have discovered the new facts that EC SOD is normally present in the dermal layer of the body skin and the expression of it is regulated by injurious UV. In addition, the present inventors have

discovered that when an EC SOD is overexpressed in a cell, active oxigen which is generated by UV is reduced. Thus, the present inventors prepared an cell-transducing fusion protein which combines a protein transduction domain to EC SOD amino terminal, and confirmed the above fusion protein was ilfliterated effectively to the skin, thereby completing the invention.

Accordingly, an object of the present invention is to provide a cell-transducing EC SOD fusion protein which combines HIV-1 Tat trnasduction region(amino acid residues 49~57), and a protein transduction domain which is selected from the groups consisting of oligopeptides constituted with 5~12 of arginin residues and oligopeptides constituted with 5~12 of lysin residues.

Another object of the present invention is to provide a polynucleotide encoding the above fusion protein.

Still another object present invention is to provide a vector comprising a polynucleotide encoding the above fusion protein.

Still another object present invention is to provide a host cell which is transformed with the above expression vector.

Still another object present invention is to provide a method for preparing a cell-transducing EC SOD fusion

protein.

Still another object present invention is to provide a pharmaceutical composition for preventing or treating ROS related diseases which contains cell-transducing EC SOD fusion protein as an effective component.

Still another object present invention is to provide a pharmaceutical composition for preventing or treating ROS related diseases which contains EC SOD fusion protein as an effective component.

Still another object present invention is to provide a pharmaceutical composition for preventing or treating ROS related diseases which contains a vector comprising a polynucleotide encoding a cell-transducing EC SOD fusion protein as an effective component.

Still another object present invention is to provide a pharmaceutical composition for preventing or treating ROS related skin diseases which contains a vector comprising a polynucleotide encoding an EC SOD fusion protein as an effective component.

Still another object present invention is to provide a cosmetic composition comprising the above cell-transducing EC SOD fusion protein.

Still another object present invention is to provide a food composition comprising the above cell-transducing EC

SOD fusion protein or EC SOD protein.

Still another object present invention is to provide a food composition comprising a microorganism which is transformed by a vector expressing the above cell-transducing EC SOD fusion protein or EC SOD protein.

【Mode for Invention】

The present invention provides a cell-transducing EC SOD fusion protein which combines HIV-1 Tat trnasduction region(amino acid residues 49~57), and a protein transduction domain which is selected from the groups consisting of oligopeptides constituted with 5~12 of arginin residues and oligopeptides constituted with 5~12 of lysin residues.

The present invention provides a polynucleotide encoding the above EC SOD fusion protein.

The present invention provides a vector comprising the above polynucleotide and a host cell which is transformed with the above expression vector.

The present invention provides a method for preparing the above fusion protein.

The present invention provides a pharmaceutical composition for preventing or treating ROS related diseases

which contains cell-transducing EC SOD fusion protein as an effective component.

In addition, the present invention provides a pharmaceutical composition for preventing or treating ROS related diseases which contains EC SOD protein as an effective component.

In addition, the present invention provides a pharmaceutical composition for preventing or treating ROS related diseases which contains a vector comprising a polynucleotide encoding a cell-transducing EC SOD fusion protein as an effective component.

In addition, the present invention provides a composition for preventing or treating ROS related skin diseases which contains a vector comprising a polynucleotide encoding EC SOD protein as an effective component.

In addition, the present invention provides a cosmetic composition comprising the above cell-transducing EC SOD fusion protein.

In addition, the present invention provides a cosmetic composition for preventing or treating ROS related skin diseases comprising the above cell-transducing EC SOD fusion protein.

In addition, the present invention provides a

cosmetic composition for preventing or treating ROS related skin diseases comprising an EC SOD fusion protein.

In addition, the present invention provides a food composition comprising a microorganism which is transformed by a vector expressing the above cell-transducing EC SOD fusion protein.

In addition, the present invention provides a food composition comprising a microorganism which is transformed by a vector expressing the above EC SOD fusion protein.

In one example of the present invention, the distribution pattern of EC SOD in the mouse skin was examined by immunohistochemical staining (see Example 1-1). The examination results could suggest that EC SOD is distributed throughout the mouse skin, and particularly, more present in the connective tissue of the dermal layer than in the epidermal layer (see FIG. 1). Also, in another example of the present invention, the expression pattern of EC SOD mRNA in the dermal and epidermal layers of the skin was examined (see Example 1-2), and as a result, a new fact that EC SOD is expressed at a much higher level in the dermal layer than in the epidermal layer could be found (see FIG. 2). From the above experimental results, it was inferred that EC SOD would perform the action of protecting the skin while it would be present in the skin tissue,

particularly the dermal layer.

Accordingly, in order to directly examine the role of EC SOD in the skin, in one example of the present invention, the mouse skin was irradiated with various UV lights (UVA, UVB and PUVA), and the expression patterns of EC SOD were analyzed at varying time points after the UV irradiation. The results could indicate that the expression of EC SOD in the skin varies depending on the presence or absence of UV irradiation (see Examples 1-3 to Examples 1-5). From the above experimental results, the present inventors have found a new fact that EC SOD is distributed throughout the skin tissue, particularly the dermal layer, while its expression varies with UV irradiation intensity and the passage of time. Accordingly, it is presumed that EC SOD has relationship with ROS which is induced by UV irradiation.

To confirm the above presumption, the present inventors examined the effect of overexpression of EC SOD on the amount of UV-induced intracellular reactive oxygen with well known gene expression system in the art by preparing a mouse EC SOD-overexpressed cell line, irradiating the cell with UV and then measuring the amount of reactive oxygen in the cell. The results could suggest

that the overexpression of mouse EC SOD effectively reduces intracellular reactive oxygen (see Example 2).

Moreover, the present inventors examined the effect of overexpression of human EC SOD on UV-induced cell death by preparing a human EC SOD-overexpressed cell line with a human keratinocyte cell line, irradiating the cell with UV and then measuring cell death. The results could indicate that the overexpression of human EC SOD effectively reduces cell death induced by UV (see Example 3).

Accordingly, EC SOD can be used in preventing or treating ROS related diseases. Particularly, EC SOD can be used in preventing or treating skin diseases. The skin diseases may be pigmentation diseases such as skin cancer, chloasma, freckles. In addition, the above EC SOD can be used in preventing or suppressing aging such as wrinkes caused by reactive oxigen and photo-aging caused by injurious uv and radiation. Accordingly, the present invention provides a pharmaceutical composition for preventing or treating ROS related skin diseases which contains an EC SOD as an effective component.

For easy application of EC SOD in a pharmaceutical composition for preventing or treating ROS related skin

diseases, the present inventors endowed an EC SOD cell-transduction ability by preparing a fusion protein which combines HIV-1 Tat trnasduction region(amino acid residues 49~57), and a protein transduction domain which is selected from the groups consisting of oligopeptides constituted with 5~12 of arginin residues and oligopeptides constituted with 5~12 of lysin residues.

Accordingly, the present invention provides a cell-transducing EC SOD fusion protein which combines HIV-1 Tat trnasduction region(amino acid residues 49~57), and a protein transduction domain which is selected from the groups consisting of oligopeptides constituted with 5~12 of arginin residues and oligopeptides constituted with 5~12 of lysin residues to amino terminal of EC SOD.

Preferably, a cell-transducing EC SOD fusion protein of the present invention has amino acid sequences represented by SEQ ID NO:12, SEQ ID NO:13, SEQ ID NO:14, and SEQ ID NO:15.

In the above mentioned, EC SOD refers a protein originated from a mammalian comprising human and mouse, preferably from human. The above human mature EC SOD is organized with 222 amino acids and it has heparin binding

domain in c-terminal(amino acid residues 210~215). The human mature EC SOD protein is defined as a protein consisting of an amino acid sequence of SEQ ID NO: 11. Furthermore, the EC SOD protein may be a protein in which the heparin transduction domain (amino acid residues 210-222) is deleted.

The protein transduction domain of the above refers to an oligopeptide consisting of several amino acid residues, which is able to introduce not only itself but also polymeric organic compounds, such as other kinds of oligonucleotides, peptides, proteins and oligosaccharides, without requiring separate receptors or energy into the cell. Examples of the protein transduction domain which can be used in the present invention include an HIV-1 Tat transduction domain, an oligopeptide consisting of 5-12 arginine residues and an oligopeptide consisting of 5-12 lysine residues. Preferably, a transduction domain of 9 amino acid residues (RKKRRQRRR) corresponding to amino acids 49-57 of HIV-1 Tat, an oligopeptide of 9 arginine residues and an oligopeptide of 10 lysine residues may be used as the protein transduction domain.

In one example of the present invention, it is confirmed that the fusion protein of the present invention can be located in the cell nuclei by transducing human keratinocyte cell from the experiment(See Example 4).

In addition, the present invention provides a polynucleotide sequence encoding EC SOD fusion protein of the above. The polynucleotide of the above is characterized in that a DNA sequence encoding HIV-1 Tat transduction domain in 5'-terminal end, one protein transduction domain which is selected from the group consisting of 5-12 arginine residues and an oligopeptide consisting of 5-12 lysine residues.

In addition, polynucleotide of the present invention is characterized in that a DNA sequence encoding 6 of histidine residues is bound to the 5'-terminal end of a protein transduction domain which is bound EC SOD.

Preferably, polynucleotide sequence encoding fusion protein of the present invention is shown in SEQ ID NO:16, SEQ ID NO:17, SEQ ID NO:18 and SEQ ID NO:19.

In addition, the present invention provides an

expression vector comprising polynucleotide sequence of the above. Preferably, expression vectors of the present invention comprise EC SOD, ΔHD/EC SOD which is deleted heparin binding domain, cDNA expressing protein transduction domain and 6 histidine, and expresssion regulating sequence thereof. More preferably, a vector of the present invention has a restriction map shown in FIG 11.

As used herein, the term "expression control sequence" refers to DNA sequences necessary for the expression of operably linked coding sequences in a certain host cell. The expression control sequences include promoters for performing transcription, optional operator sequences for controlling the transcription, sequences encoding suitable mRNA ribosome-binding sites, and transcriptional and translational termination regulatory sequences. For example, control sequences suitable for prokaryotic organisms include promoters, operator sequences, and sequences encoding ribosome-bind sites. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

Furthermore, the present invention provides a microorganism which is transformed with expression vector of above. As the host microorganisms, any microorganisms

can be used without special limitations if they can express the cell-transducing EC SOD fusion protein of the present invention. Examples of such host microorganism include bacteria, such as *E. coli*, bacillus, Pseudomonas and Streptomyces, eukaryotic and prokaryotic host cells, such as fungi and yeasts, insect cells, such as *Spodoptera frugiperida* (SF9), animal cells, such as CHO and mouse cells, and tissue-cultured human and plant cells. Preferred microorganisms include bacteria such as *E. coli* and *bacillus subtilis*. Depending on applications, microorganisms for food proved to be safe in the human body, such as lactic acid bacteria, may be used.

In addition, the present invention provides a method for preparing cell-transducing EC SOD fusion protein which is prepared by the steps of: (a) transforming a host microorganisms with a recombinant expression vector comprising a polynucleotide sequence encoding the cell-transducing EC SOD fusion protein; (b) culturing the transformed microorganisms prepared in the step (a) in a suitable medium and condition for the expression of the polynucleotide sequence; and (c) collecting a substantially pure fusion protein encoded by the polynucleotide sequence from the culture solution of the step (b).

In the present invention, "substantially pure" refers that a fusion protien of the present invention substantially does not comprise protiens which is originated form bacteria.

The present invention provides a pharmaceutical composition for preventing or treating ROS related diseases containing a cell-transducing EC SOD fusion protein as an effective component.

In addition, the present invention provides a pharmaceutical composition for preventing or treating ROS related skin diseases containing an EC SOD fusion protein as an effective component. An EC SOD protein of the above may be prepared with genetic engineering method by well known EC SOD nucleic acid sequences in the art.

In the present invention, "ROS(Reactive Oxigen Species) related diseases" refers the diseases caused by ROS. In the present invention, particularly, it refers the skin diseases caused by ROS, for example, pigmentation diseases such as skin cancer, chloasma, freckles. It may also be skin aging, photo-aging caused by injurious UV or irradiation, prevention and treatment of dermatitis.

The inventive pharmaceutical composition may comprise a pharmaceutically acceptable carrier together with the EC SOD fusion protein or the EC SOD protein. As used herein, the term "pharmaceutically acceptable carrier" refers to a substance that is physiologically acceptable and does not generally cause allergic reactions, such as gastrointestinal disorder and dizziness etc. or reactions similar thereto when administered into humans.

The pharmaceutically acceptable carriers may include, for example, carriers for oral administration or for parenteral administration. The carriers for oral administration may include lactose, starch, cellulose derivatives, magnesium stearate, stearic acid and so on. Also, the carriers for parenteral administration may include water, suitable oils, saline solution, aqueous glucose and glycol etc. and the inventive composition may further comprise stabilizers and conservatives. Suitable stabilizers include antioxidants, such as sodium bisulfite, sodium sulfite or ascorbic acid. Suitable preservatives include benzalkonium chloride, methyl- or propyl-paraben, and chlorobutanol. For other pharmaceutically acceptable carriers, reference may be made to the following literature: Remington's Pharmaceutical Sciences, 19th ed.,

Mack Publishing Company, Easton, PA, 1995.

The cell-tranducing EC SOD fusion protein or the EC SOD protein, which is contained in the inventive composition, may be formulated into various parenteral or oral dosage forms, together with the pharmaceutically acceptable carrier as described above. The formulations for parenteral administration preferably include injection formulations, such as isotonic aqueous solution or suspension formulations, and ointment formulations. The injection formulations may be prepared using suitable dispersing or wetting agents, and suspending agents, according to the known methods in the art. For example, each ingredient is dissolved in saline or buffer and then can be prepared into a dosage form for injection. Examples of the formulations for oral administration include, but are not limited to, powders, granules, tablets, pills and capsules, and these formulations may comprise, in addition to the active ingredient, diluents (e.g., lactose, dextrose, sucrose, mannitol, sorbitol, cellulose, and/or glycin) and lubricants (e.g., silica, talc, stearic acid, and magnesium or calcium salts thereof, and/or polyethylene glycol). The tablets may comprise binders, such as magnesium aluminum silicate, starch paste, gelatin, tragacanth, methyl cellulose, sodium carboxymethylcellulose and/or

polyvinylpyrrolidone, and if necessary, it may further comprise disintegrants, such as starch, agar-agar, alginate or a sodium salt thereof, or azeotropic mixtures and/or absorbing agents, coloring agents, flavoring agents and sweetening agents. These formulations may be prepared by a conventional mixing, granulation or coating method.

Routes for the administration of the inventive pharmaceutical composition include, but are not limited to, oral, intravenous, intramuscular, intraarterial, intramarrow, intrathecal, intracardiac, transdermal, epicutaneous, subcutaneous, intraperitoneal, intranasal, intestinal tract, local, sublingual and rectal routes.

Preferably, the pharmaceutical composition according to the present invention can be administered parenterally by a subcutaneous, intravenous, intramuscular, intraarticular, intrabursal, intrasternal, intrathecal, intralesional or intracranial injection or infusion technique. For example, the inventive pharmaceutical composition formulated for injection may be administered by messotherapy which is the method of injection into the skin at a given amount by a fine needle of 4-6 mm or lightly pricking the skin with a 30-gage injection needle. Also, for application to the skin, the inventive pharmaceutical composition may be formulated into, for example, ointment

preparations, in the transdermal or epicutaneous administration. As used herein, the term "transdermal or epicutaneous administration" means that the pharmaceutical composition is locally administered into the skin such that an effective amount of an active ingredient contained in the pharmaceutical composition is transferred. Particularly, a pharmaceutical composition containing the inventive cell-transducing EC SOD fusion protein as an active ingredient is preferably administered by the transdermal or epicutaneous administration technique for direct application to the skin. Furthermore, the inventive pharmaceutical composition may be administered by a bioengineering technique associated with a protein transduction method.

The pharmaceutical composition according to the present invention may be administered to a patient at an amount showing a preventive or therapeutic effect. Generally, this EC SOD protein may be administered at a daily dose ranging from about 0.0001 to 100 mg/kg, and preferably about 0.01 to 1 mg/kg. The inventive pharmaceutical compositions may be administered at an amount within the preferred daily dose range one time or several times each day. However, the dose of the inventive

pharmaceutical composition may be suitably selected according to an administration route, a subject to be administered, and the age, sex, body weight, characteristic and disease condition of the subject.

In addition, the present invention provides a pharmaceutical composition for preventing or treating ROS related diseases containing an expression vector comprising polynucleotide encoding an cell-transducing EC SOD fusion protein of the present invention as an effective component. The ROS related diseases are described above.

Furthermore, the present invention provides a pharmaceutical composition for preventing or treating ROS related skin diseases containing an expression vector comprising polynucleotide encoding an EC SOD protein of the present invention as an effective component. The ROS related diseases are described above.

The expression vector of above may be plasmid or virus vector, and the vector refers to a vector which can introduce phenotype into the target cell by the method of infection or transduction which are well known in the art.

A plasmid expression vector is a means for transferring plasmid DNA directly to human cells by a gene

transfer method approved by FDA for use in human beings (Nabel EG, et al., *Science*, 249:1285-1288, 1990). Plasmid expression vectors which can be used in the present invention include mammalian expression plasmids known in the art. Typical examples include, but are not limited to, pRK5 (European Patent No. 307,247), pSV16B (PCT publication No. 91/08291) and pVL1392 (PharMingen).

The plasmid expression vector may be introduced into a target cell by any method known in the art, such as transient transfection, microinjection, transduction, cell fusion, calcium phosphate precipitaiton, liposome-mediated transfection, DEAE dextran-mediated transfection, polybrene-mediated transfection, electroporation, gene gun or other methods for introducing DNA into cells (Wu et al., *J Biol Chem*, 267:963-967, 1992; Wu and Wu, *J Biol Chem*, 263:14621-14624, 1988).

Moreover, the virus expression vectors according to the present invention include, but are not limited to, retrovirus, adenovirus, herpes virus and avipox virus.

The virus vector may be administered by any method known in the art. For example, it may be administered by a local, parenteral, oral, intranasal, intravenous, intramuscular or subcutaneous route or any other suitable

route. Particularly, the vector may be injected directly into a target cell in an effective amount.

In another further aspect, the present invention provides a cosmetic composition comprising the cell-transducing EC SOD fusion protein of the present invention. The inventive cosmetic composition may be very effectively used against aging or pigmentation diseases, such as, melasma and freckles. The aging diseases include all natural aging and photoaging. The natural aging refers to aging that naturally occurs with advancing years, and the photoaging refers to aging that occurs by artificial or natural exposure to UV, etc. In addition, the inventive cosmetic composition is effective in the prevention and improvement of wrinkles caused by aging.

The functional cosmetic composition according to the present invention may be prepared by a conventional method. The inventive EC SOD protein may be contained in an amount of 0.001-50% by weight, and preferably 0.1-20% by weight, based on the dry weight of the cosmetic composition.

Along with dermatologically acceptable carriers, the inventive cosmetic composition may be applied in foundation cosmetic compositions (e.g., toilet water, cream, essence, cleansing foam, cleansing water, pack, and body oil), color

cosmetic compositions (e.g., foundation, lipstick, mascara, and make-up base), and the like. Such carriers may include skin softner, skin penetration enhancers, coloring agents, aromatics, emulsifiers, thickeners, and solvents.

Furthermore, the present invention provides a food composition comprising cell-transducing EC SOD fusion protein of the present invention. In addition, the present invention provides a food composition comprising EC SOD protein of the present invention. The above food composition may be used for preventing or treating ROS related disease, particularly ROS related skin disease.

The food composition of the present invention may be prepared as a form of health food composition of cell-transducing EC SOD fusion protein or EC SOD protein with sitologically acceptable carriers. For example, a form of tablet, camsule, powder, granule, liquid, or pill may be manufactured by admixing cell-transducing EC SOD fusion protein or EC SOD protein with a diluent, excipient and the like according to the method known in the art.

In addition, the present invention provides a food composition containing a microorganism transformed with an expression vector comprising polynucleotides endocing cell-

transducing EC SOD fusion protein or EC SOD as an effective ingredient.

The above microorganism may be administered by various routes. For example, effective amount of the above microorganisms may be prepared by admixing with a pharmaceutically acceptable carrier. As a carrer, for example, a form of tablet, troches, capsules, elixirs, suspensions, syrups, wafer, powders, or granules can be used with admixing binder, lublitant, solutionizer, exipient, solubilizer, dispersing agent, stabilizer, suspending agent, colorant and flavor to it. Preferable administration route is oral administration. In other aspect, a pharmaceutical composition of the present invention may be prepared into various forms such as micro capsulation which is known in the art with the method known in the art. The dose of the composition may be suitably selected according to absorbtion rate of the active ingredient into the body, inactivation rate and excretion rate, age, sex and status of the patient, and disease condition.

Hereinafter, the present invention will be described in detail by way of the following examples. It is to be understood, however, that these examples are given for

illustrative purpose only and are not intended to limit the scope of the present invention.

Example 1: Examination of distribution pattern of EC SOD in mouse skin

1-1) Examination of distribution pattern of EC SOD in mouse skin using immunohistochemical staining

In order to examine the distribution of EC SOD in skin tissue, immunohistochemical staining was performed. The skin tissue taken from BALB/C mice (8 to 10-week-old, female, obtained from KRIBB, Taejon, Korea) was fixed in 4% paraformaldehyde and prepared into a paraffin block by a known method. The paraffin block was treated with xylene to remove paraffin, and dehydrated by treatment with alcohol. To enhance an immune response, the tissue sample was then thermally treated in citric acid buffer at 121 °C for 10 minutes. Next, the tissue sample was treated with 3% hydrogen peroxide (H_2O_2) to inhibit peroxidase, and reacted with a rabbit anti-mouse EC SOD antibody (primary antibody) diluted 1:500, for 60 minutes. The rabbit anti-mouse EC SOD antibody was obtained by injecting mouse EC SOD into rabbits to immunize the rabbits. The tissue sample reacted with the antibody was washed with 0.1M PBS

and reacted with 200 μl of a biotin-conjugated goat anti-rabbit IgG-containing antibody (Universal LSAB 2 kit, Dako, Glostrup, Denmark) for 15 minutes. Also, pre-immune rabbit serum was used as a negative control group. After completion of the reaction, the tissue sample was washed with PBS, reacted with peroxidase-conjugated streptavidin, and color-developed with 3-amino-9-ethyl-carbazole (AEC, chromogen, Utah, USA), a substrate solution. The developed tissue sample was prepared into a test sample by a conventional method and observed under a microscope at a magnification of 400X.

The test results revealed that EC SOD was distributed throughout the skin tissue. It was observed that EC SOD was widely distributed in the connective tissue of the dermal layer, and particularly, it was more strongly stained around the hair follicles and blood vessels of the dermal layer. Also, EC SOD was strongly stained also in the cells of the epidermal layer, and thus, it was inferred that EC SOD of these region of skin would have the activity associated scavenging roles to reactive oxygen species (see FIG. 1).

1-2) Examination of expression pattern of EC SOD in mouse skin using Northern blot analysis

The ear skin tissue of BALB/C mice in Example 1-1) was separated by cutting with surgical scissors and rinsed with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (pH 7.4). The skin tissue was floated with the dermis side down on 0.5% trypsin (Gibco, Invitrogen Corporation, California, U.S.A) in PBS at 37 °C for 50 minutes while the dermal layer faced downward. Then, the epidermal layer was separated from the dermal layer.

Total RNA was isolated from each of the separated epidermal and dermal layers. 20 μg of the extracted RNA was electorphoresed in formaldehyde-containing 1.2% agarose gels, and the mRNA on the gel was transferred to a nylon membrane, after which the RNA attached to the membrane was crosslinked by UV. A ^{32}P-labeled cDNA probe of EC SOD was hybridized to the membrane at 65 °C. The cDNA of EC SOD was prepared by PCR-amplification using the pCRII TOPO vector (provided from professor Suh JG, College of Medicine, Hallym University) (Suh JG et al., *Mol Cells*. 30; 7(2), 204-7, 1997) containing a mouse skin EC SOD cDNA as a template and the following primers. The PCR amplification reaction consisted of the following: one cycle at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 55 °C, and 45 sec at 72.5 °C; and one cycle of 5 min at 72.5 °C.

Sense primer (SEQ ID NO: 1):

5'-ATG TTG GCC TTC TTG TTC-3'

Anti-sense primer (SEQ ID NO: 2):

5'-TTA AGT GGT CTT GCA CTC-3'

After completion of the hybridization, the membrane was washed and exposed to a film. The exposed film was developed to confirm mRNA, and analyzed with a molecular imager (Image Master-VDS, Phamacia Biotech). A significant difference between the test groups was assayed with Student's t-test.

The results showed that the expression of EC SOD mRNA was about 7 times stronger in the dermal layer than in the epidermal layer (see FIG. 2). From the test results, it could be inferred that EC SOD is strongly expressed in the fibroblasts and endothelial cells of the dermal layer. From such results, it was suggested that EC SOD would protect the skin, and particularly, it would have high activity of protecting the dermal connective tissue from reactive oxygen species.

1-3) Examination of expression pattern of EC SOD in mouse skin after irradiation with UVA

In order to directly examine the role of EC SOD in the *in vivo* skin, the dorsal skin of mice was irradiated with UVA, the total RNA was isolated from the dorsal skin of mice at different time points after UVA irradiation and subjected to Northern blot analysis to investigate the effect of UVA irradiation on the expression pattern of EC SOD.

The BALB/C mice were divided into each group of three animals. Theirs backs were shaved and after 24 hours, irradiated with different intensities of UVA. The mice were irradiated at an intensity of $5kJ/m^2$ and $25kJ/m^2$ radiated at an intensity of by means of 6 UVA lamps (F24T12/BL/HO, National Biological corporation USA) emitting $9.7mW/cm^2$, respectively. A control group was not irradiated with UVA.

The skin tissues were taken from UVA irradiated mice at indicated time points. From the separated skin tissue, total RNA was isolated and then subjected to Northern blot analysis in the same manner as in Example 1-2) to investigate the expression pattern of EC SOD mRNA. The amount of expressed mRNA was quantitatively analyzed using a molecular imager. As a loading control, GAPDH was used. The results were showed as mean ± standard deviation, and the expression level of each of the test groups was showed

as a value relative to the expression level of the control group taken as 100%. Also, a significant difference between the control group and the test groups was assessed with Student's t-test.

As the result, the expression of EC SOD was not influenced by an UVA irradiation of 5 kJ/m^2. However, when the mice were irradiated with UVA of 25 kJ/m^2, the expression of EC SOD was initially reduced, and 6 hours after the UV irradiation, reached a maximum point of 338 ± 7.2% ($p<0.05$), and then, 96 hours after the UV irradiation, showed a tendency to decrease (see FIG. 3). Thus, it could be found that the expression of EC SOD is controlled according to the intensity of UVA and the elapsed time after the UVA irradiation. From such results, it was inferred that EC SOD would perform a function of protecting the skin tissue from reactive oxygen species overproduced after UVA irradiation and changes caused thereby.

1-4) Examination of expression pattern of EC SOD in mouse skin after irradiation with UVB

The dorsal skin of mice was irradiated with UVB, and then, RNA was extracted from the irradiated skin at different time points and subjected to Northern blot analysis to investigate the effect of UVB irradiation on

the expression pattern of EC SOD. According to the same method as described in Example 1-3), the BALB/C mice were divided into each groups of three animals, and then they were irradiated with $2kJ/m^2$, $8kJ/m^2$ and $15kJ/m^2$, respectively, by means of 6 UVB lamps (FS24T12/UVB/HO, National Biological corporation, USA) emitting $0.6mW/cm^2$. After the UV irradiation, the expression pattern of mRNA was examined in the same manner as in Example 1-2).

As the result, when the mice were irradiated with $2kJ/m^2$ of UVB, the expression of EC SOD mRNA was initially reduced to 73 ± 3% ($p<0.05$), but after 24 hours, restored to the level of the control group. When the mice were irradiated with 8 kJ/m^2 of UVB, the expression of EC SOD was increased to 212 ± 11% ($p<0.05$) after 48 hours. In the case of the irradiation at an intensity of 15 kJ/m^2, the expression level of EC SOD was increased to 322 ± 15% ($p<0.05$) after 48 hours, which is the highest value (see FIG. 4). Accordingly, it could be found that the expression of EC SOD is influenced by the intensity of UVB and the elapsed time after UV irradiation.

1-5) Examination of expression pattern of EC SOD in mouse skin after treatment with PUVA

Mice were treated with PUVA which is widely used in

clinical treatment. Then, RNA was isolated from the treated mice at the different time points and subjected to Northern blot analysis to investigate the effect of PUVA treatment on the expression pattern of EC SOD.

According to the same method as described in Example 1-3), two test groups each consisting of three BALB/C mice were applied with 100 μl of 8-MOP (0.2% (w/v) 8-methoxy psolaren, ICN Pharmaceuticals, Costa Mesa, USA) on their dorsal skin, and after one hour, irradiated with UVA at intensities of 5kJ/m^2 and 25kJ/m^2, respectively, by means of the same UV lamps as used in Example 1-3). At this time, a control group was treated only with 0.2% (w/v) 8-MOP. After the UV irradiation, the expression pattern of EC SOD mRNA was examined in the same manner as in Example 1-2).

As the result, in the case of the control group treated with 8-MOP only and in the case of the test group treated with 8-MOP followed by irradiation with 5 kJ/m^2 of UVA, there was no change in the expression level of EC SOD mRNA. In the case of the test group irradiated with 25kJ/m^2 of UVA, the expression level of EC SOD mRNA was increased slowly after the UV irradiation and reached 264 ± 4.5% ($p<0.05$) after 48 hours, indicating that the expression level of EC SOD was remarkably increased.

From the above test results, it could be found that the expression pattern of EC SOD is influenced by the intensity of UV and the elapsed time after UV irradiation. Accordingly, it was inferred that EC SOD would have an association with the activity of protecting the skin from toxic reactive oxygen species produced either after UV irradiation or in an inflammatory reaction caused by UV.

Example 2: Change in reactive oxygen in mouse EC SOD-overexpressed cell line according to UV irradiation

EC SOD was overexpressed in mouse embryonic fibroblasts using a Tet off-MEF/3T3 inducible gene expression system (Clontech), a known gene expression system, after which the cells were irradiated with UV and measured for intracellular reactive oxygen.

2-1) Construction of mouse EC SOD-overexpressed cell line

Mouse EC SOD cDNA was inserted into the Sal I site of a TRE2 vector (Clontech), a control vector of a TET OFF system. The mouse EC SOD was prepared by PCR-amplification using pCRII TOPO vector (provided from professor Suh, JG, College of Medicine, Hallym University) containing mouse EC

SOD cDNA as a template with the following primers. The PCR amplification reaction consisted of the following: one cycle at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 55 °C, and 45 sec at 72.5 °C; and one cycle of 5 min at 72.5 °C.

Sense primer (SEQ ID NO: 3):

5'-AGT CTC GAG ATG TTG GCC TTC TTG TTC TAC GGC-3'

Anti-sense primer (SEQ ID NO: 4)

5'-GATC CTC GAG TGG TCT TGC ACT CGC TCT-3'

The prepared vector and a hygromycin-resistant sequence-containing pTG76 plasmid (University of Geneva Medical School, Geneva, Switzerland) were introduced into mouse embryonic fibroblasts (Clontech), a MEF3T3/TET OFF cell line, using lipofectin (Invitrogen). Then, the cells were cultured in a medium containing 100 μg/mL hygromycin, and only resistant colonies were selected. Of the colonies, a cell line overexpressing the EC SOD upon the removal of tetracycline was constructed. Namely, the colonies were cultured in a DMEM medium containing 10% TET-free FBS (Clontech), L-glutamine, 5000 UI/L penicillin streptomycin, 100 μg/mL G418, 100 μg/mL hygromycin and 2ng/mL tetracycline and then tetracycline was removed in order to

overexpress EC SOD. The removal of tetracycline was performed by culturing the cells in a fresh medium free of tetracycline. The cells were cultured in the fresh medium for 48 hours so as to induce the overexpression of EC SOD and measured for the expression level and activity of EC SOD. The expression level of EC SOD was measured by Northern blot analysis in the same manner as in Example 1-2), and the activity of EC SOD was analyzed by adding 20 μl of the medium sample and 10 μl of xanthine oxidase (SIGMA) to 3 ml of 50mM sodium carbonate buffer (containing 0.1 mM cytochrome C, and 0.5 mM xanthine, pH 10.0) and then measuring the absorbance at 550nm.

In the result, when tetracycline was removed, the expression and activity of EC SOD mRNA showed a tendency to increase with the passage of time. Thus, a cell line overexpressing the mouse EC SOD could be constructed.

2-2) Measurement of reactive oxygen in mouse EC SOD-overexpressed cell line after UV irradiation

The mouse EC SOD-overexpressed cell line constructed in Example 2-1) was irradiated with UV and then measured for the amount of reactive oxygen. Before the UV irradiation, the media were removed and the mouse EC SOD-

overexpressed cell was washed two times with PBS (pH 7.4), and irradiated with UV in the presence of PBS. The UV irradiation was performed by irradiating the cell line with 10 J/cm^2 of UVA by means of an UVA lamp emitting 27 mW/cm^2, and irradiating the cell line with 20mJ/cm^2 of UVB by means of three UVB lamps emitting 1.15 mW/cm^2. The PUVA treatment was performed by treating the cell line with 0.1% 8-MOP and, after 30 minutes, washing the treated cell line with PBS two times and irradiating with 2J/cm^2 of UVA.

After irradiating the cells with UV as described above, the cells were sampled at different time points and reacted with 10 μM HE (dihydroethyidium) at 37 °C for 20 minutes. The resulting sample was washed with PBS containing 1%(w/v) BSA and 0.1%(w/v) NaN_3 and was analyzed with a flow cytometer. A value measured with the flow cytometer was showed as a value relative to the reactive oxygen species amount of the control group taken as 1. As the control group, a cell line which has not been irradiated with UV was used.

The results showed that, when the mouse EC SOD-overexpressed cell line was irradiated with 10J/cm^2 of UVA, the amount of reactive oxygen species was reduced continuously for 6 hours after the UV irradiation (see FIG. 7A). Also, when the EC SOD-overexpressed cell line was

irradiated with 20mJ/cm^2 of UVB, the concentration of reactive oxygen species was about 60% reduced one hour after the UV irradiation as compared to the control cell line (see FIG. 7B). Also, when the EC SOD-overexpresed cell line was treated with PUVA, the amount of intracellular reactive oxygen was dramatically reduced as compared to the EC SOD-nonoverexpressed cell line (see FIG. 7C).

From the above results, it could be found that EC SOD not only functions in serum and extracellular matrices but also has the activity of effectively reducing the concentration of reactive oxygen species in cells.

Example 3: Effect of UV irradiation on cell death in human EC SOD-overexpressed cell line

3-1) Construction of human EC SOD-overexpressed cell line

In order to examine a change in reactive oxygen in a human EC SOD-overexpressed cell line according to UV irradiation, a human EC SOD-expressed cell line was constructed. For this purpose, human EC SOD cDNA was first inserted into the XbaI and EcoRI sites of pcDNA 3.1/myc-His(A) (Invitrogen). The human EC SOD cDNA was prepared by

PCR-amplification using pUC18-hEC SOD vector (provided from professor Marklund, Clinical chemistry, Sweden) (Karin Hjalmarsson, *Proc Natl Acad Sci* USA Vol.84, 6340-4, 1987) containing an human EC SOD cDNA as a template with the following primers. The PCR amplification reaction consisted of the following: one cycle of 4 min at 98℃; 30 cycles of 30 sec at 98 ℃, 30 sec at 55 ℃, and 45 sec at 72 ℃; and one cycle of 5 min of 72 ℃.

Sense primer (SEQ ID NO: 5):

5'-ATC TCT AGA ATG CTG GCG CTA CTG TGT-3'

Anti-sense primer (SEQ ID NO: 6)

5'-ATC GAA TCC TCA GGC GGC CTT GCA CTC GCT CTC -3'

The resulting PCR product was separated by agarose gel electrophoresis, treated with XbaI and EcoRI enzymes, and the resulting product inserted into a pcDNA 3.1/myc-His(A) vector (see FIG. 8). The resulting plasmid was transformed into HaCaT cells (provided from Professor N.E. Fusenig, The University of Heidelberg, Germany), a human keratinocyte cell line, using lipofectin (Invitrogen). Then, the transformed cells were cultured in a 500 μg/ml

neomycin (Gibco)-containing media, and only resistant colonies were selected, thus constructing an EC SOD-overexpressed cell line.

In order to confirm the overexpression of EC SOD in the cells, Western blot analysis was performed. The EC SOD-overexpressed cell line was reacted with PBS containing 1% NP-40, 0.1% SDS and protease inhibitor, followed by centrifugation, and the supernatant was collected, thus extracting a protein from the EC SOD-overexpressed cell line. The extracted protein was subjected to 15% SDS-PAGE, moved to a nitrocellulose membrane, and blocked with 5% non-fat milk/TBS-0.1% Tween 20 solution for 1 hour. Thereafter, the resulting protein was reacted with a HIS antibody (Santa Cruz) diluted 200:1, for one hour, and washed three times with TBS-0.1% Tween 20 solution. Next, the reaction material was reacted with peroxidase-conjugated anti-rabbit IgG for 1 hour and washed three times, after which the expression of EC SOD was examined with an ECL kit (Amersham Bioscience). Also, a protein of a non-transformed HaCaT cell line was used as a negative control.

The results showed that, in the negative control HaCaT cell line, the histidine-bound EC SOD was not detected, but in the cell line transformed with the EC SOD

cDNA-containing plasmid, the histidine-bound EC SOD was detected, thus indicating that EC SOD was overexpressed in the transformed cell line (see FIG. 9).

3-2) Measurement of cell death in human EC SOD-overexpressed cell line after UV irradiation

The human EC SOD-overexpressed cell line constructed in Example 3-1) was UV-irradiated and then measured for the amount of reactive oxygen species. Before the UV irradiation, the media were removed and human EC SOD-overexpressed cell line was washed twice with PBS (pH 7.4), and irradiated with UVB in the presence of PBS. The UV irradiation was performed by irradiating the cell line with each of 10, 20 and 30 mJ/cm^2 of UVB by means of three UVB lamps emitting $1.15mW/cm^2$. After 24 hours, cells were sampled, washed with PBS (pH 7.4), and fixed in 80% ethanol. The cells were precipitated by centrifugation, washed with PBS (pH7.4), suspended in 1 ml PBS (pH 7.4), and reacted with 4 ml of permeabilization solution (0.5% Tripton X-100, 200 μg/ml RNase A, 10 μg/ml propidium iodide) for 15 minutes. Cell cycle analysis was performed with a flow cytometer, and apoptototic cells (Sub G1) were showed as percentage. At this time, a HaCaT cell line which has not been transformed was used as a negative control group.

In the result, when UVB was irradiated at an intensity of 10mJ/cm^2, the percentage of apoptototic cells did not show a great difference between the control group cell line and the EC SOD-overexpressed cell line. However, when UVB was irradiated at intensities of 20 and 30mJ/cm^2, the percentage of apoptototic cells was significantly lower in the EC SOD-overexpressed cell line than in the control group (see FIG. 10).

From the above results, it could be found that the overexpression of EC SOD can reduce cell death caused by UV in the human keratinocyte cell line. This suggests that the overexpression of EC SOD can protect skin cells from UV damages.

Example 4: Preparation of cell-transducing human EC SOD fusion protein

4-1) Preparation of TAT-EC SOD fusion protein, R9-EC SOD fusion protein and K10-EC SOD fusion protein expression vectors

Fusion proteins where a human EC SOD protein had been fused to protein transduction domains, a HIV-1 Tat (RKKRRQRRR), an oligopeptide consisting of 9 arginine residues, and an oligopeptide consisting of 10 lysine

residues respectively, were prepared. Also, a fusion protein where a ΔHD/EC SOD protein deleted a heparin domain from the EC SOD had been fused to HIV-1 Tat was prepared.

For this purpose, human EC SOD and ΔHD/EC SOD cDNAs were first prepared. The human EC SOD c DNA was prepared by PCR-amplification using pUC18-hEC SOD vector (provided from professor Marklund, Clinical chemistry, Sweden) containing a human EC SOD cDNA as a template and the following primers (SEQ ID NO: 7 and SEQ ID NO: 8). The PCR amplification reaction consisted of the following: one cycle of 4 min at 98 °C ; 30 cycles of 30 sec at 98 °C , 30 sec at 55 °C , and 45 sec at 72 °C ; and one cycle of 5 min at 72 °C.

Sense primer (SEQ ID NO: 7):

5'-GAT CCT CGA GTG GAC GGG CGA GGA CTC GGC-3'

Anti-sense primer (SEQ ID NO: 8):

5'-GAT CCT CGA GTC AGG CGG CCT TGC ACT CGC T-3'

In the case of the ΔHD/EC SOD deleted a heparin domain from the EC SOD, PCR amplification was performed under the same conditions as described above except that

the following primers (SEQ ID NO: 9 and SEQ ID NO: 10) were used.

Sense primer (SEQ ID NO: 9):

5'-GAT CCT CGA GTG GAC GGG CGA GGA CTC GGC-3'

Anti-sense primer (SEQ ID NO: 10)

5'-AAT GCT CGA GTC ACT CTG AGT GCT CCC GCG C-3'

The resulting PCR products were separated by agarose gel electrophoresis and treated with XhoI enzyme. Each of the enzyme-treated mature EC SOD cDNA and mature ΔHD/EC SOD cDNA was inserted into the XhoI site of a pET15(b)-TAT vector (provided from professor Choi SY, Department of Genetic Engineering, Hallym University) containing the fundamental domain of HIV-1 Tat (amino acid residues 49-57: RKKRRQRRR) (Park et al., *J Gen Virol*, 83:1173-1181, 2002), thus preparing the respective expression vectors. Also, the enzyme-treated mature EC SOD cDNA was inserted into the XhoI site of each of a pET15(b)-R9 vector (provided from professor Choi SY, Department of Genetic Engineering, Hallym University) containing an oligopeptide consisting of 9 arginine residues (Ryu et al., *Mol Cells*, 16:385-391, 2003) and a pET15(b)-K10 vector (provided from professor

Choi SY, Department of Genetic Engineering, Hallym University) containing an oligopeptide consiting of 10 lysine residues, thus preparing the respective expression vectors (see FIG. 11).

4-2) Transformation of *E. coli* and expression of fusion protein

E coli BL21 (DE3) (Novagen) was transformed with each of the expression vectors prepared in Example 4-1) by a heat shock method. Then, the transformed *E. coli* strain was inoculated in 250 ml of an ampicillin-containing LB medium and cultured at 37 °C to an A_{600} of 0.8 while stirring at 200 rpm. The culture solution was added with IPTG (1mM) and further cultured so as to induce the overexpression of the EC SOD fusion protein.

4-3) Purification of fusion proteins

The cells cultured in Example 4-2) were collected by centrifugation, suspended in binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, 6 M urea, pH 7.9), and disrupted by sonication. Since each of the fusion proteins contains 6 histidines at the N-terminal end, it can be purified with a very high purity of about 90% by immobilized metal-chelate affinity chromatography. For

this reason, the cell disruption solution was centrifuged, and the collected supernatant was immediately loaded into a 2.5ml Ni^{2+}-nitrilotriacetic acid sepharose column and washed with a ten-fold volume of binding buffer and a six-fold volume of washing buffer (30 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl, (pH 7.9)), after which the fusion protein was eluted with elution buffer (1 M imidazole, 0.5 M NaCl, 20 mM Tris-HCl, (pH7.9)). Next, fractions containing each of the fusion proteins were collected and desalted by performing Sephadex G-25 column (PD 10 column, Amersham Biotech) chromatography. The purified TAT-EC SOD fusion protein, TAT-ΔHD/EC SOD fusion protein, K10-EC SOD fusion protein and R9-EC SOD fusion protein were identified by electrophoresis (see FIGS. 12 and 13)

4-4) Examination of cell-transduction ability of fusion proteins

The cell-transduction ability of the inventive TAT-EC SOD fusion protein, TAT-ΔHD/EC SOD fusion protein, K10-EC SOD fusion protein and R9-EC SOD fusion protein was examined using a human keratinocyte cell line. Namely, HaCaT cells were seeded onto a slide chamber, and after 24 hours, treated with the EC SOD fusion proteins prepared according to the present invention, at concentrations of

each of 0, 1, 2 and 4 μM. After 3 hours, the resulting substance was fixed in 3.7% formaldehyde/PBS (pH 7.4) for 10 minutes and incubated with 0.1% triton X-100/PBS for 10 minutes to make them permeable. Then, it was blocked with 2% (v/v) FCS/PBS at ambient temperature for one hour, reacted with HIS antibody/2% FCS/PBS/PBS (Santacruz, USA) diluted 100:1, for 1 hour, and washed three times with 2% FBS/PBS. The resulting substance was reacted with FITC-conjugated IgG (Serotec) diluted 50:1, for 1 hour. The reaction substance was reacted with 1 μg/ml PI for 15 minutes and washed three times with 2% FBP/PBS. Then, it was mounted and observed with a confocal microscopy. As a negative control group, an EC SOD protein which has not been fused with the protein transduction domain was used.

Also, from the HaCaT cells treated with the TAT-EC SOD fusion protein or the TAT-ΔHD/EC SOD fusion protein, a protein was extracted and subjected to Western blot analysis using the HIS antibody. The intensity of the analyzed bands was quantified and showed as percentage relative to the control group EC SOD protein to which the transduction domain has not been fused. Furthermore, the cell penetration efficiencies of the K10-EC SOD fusion protein and the R9-EC SOD fusion protein were analyzed by Western blot analysis and showed as percentage relative to

the cell transduction efficiency of the TAT-EC SOD fusion protein.

The results showed that the negative control group could not penetrate into the cells, whereas the TAT-EC SOD fusion protein effectively transduced into the cells so that it was located within the cytoplasm. Also, the TAT-ΔHD/EC SOD fusion protein transduced into the cells so that it was located within the cytoplasm (see FIG. 14).

Moreover, the results of calculation of cell penetration rate showed that the cell transduction efficiencies of the TAT-EC SOD fusion protein and the TAT-ΔHD/EC SOD fusion protein were increased with an increase by concentration dependent manner with the fusion protein, and the cell transduction efficiency of the TAT-EC SOD fusion protein was higher than that of the TAT-ΔHD/EC SOD fusion protein (see FIG. 15).

Also, the R9-hEC SOD fusion protein where an oligopeptide consisting of 9 arginine residues has been fused to EC SOD showed the highest transduction efficiency. Next to the R9-hEC SOD fusion protein, the cell transduction efficiency was higher in the order of the K10-hEC SOD fusion protein having an oligopeptide consisting of 10 lysine residues fused to hEC SOD, and the TAT-EC SOD fusion protein (see Table 1).

Table 1: Cell transduction efficiency of EC SOD fusion protein

EC SOD fusion protein	transduction efficiency (%)
TAT-hEC SOD	100
R9-hEC SOD	160
K10-hEC SOD	150

【 Advantageous Effect】

An EC SOD of the present invention has the effect of reducing ROS in skin cell, particularly, Cell-transducing EC SOD fusion protein is excellent in cell transduction, and ROS reduction in a cell, therefore, it may be useful in preventing and treating ROS related disease.

【 Claims 】

Claim 1

A cell-transducing EC SOD fusion protien which combines HIV-1 Tat trnasduction region(amino acid residues 49~57), and a protein transduction domain which is selected from the groups consisting of oligopeptides constituted with 5~12 of arginin residues and oligopeptides constituted with 5~12 of lysin residues.

Claim 2

The cell-transducing EC SOD fusion protien of Claim 1, wherein the protien has a amino acid sequence which is selected from the group consisting of sequences represented by SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14 and SEQ ID NO: 15.

Claim 3

The polynucleotide of Claim 1 or 2, wherein the polypeptide encodes fusion protein.

Claim 4

The polynucleotide of Claim 3, wherein the polynucleotide has nucleotide sequence which is selected from the group consisting of sequences represented by SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18 and SEQ ID NO: 19.

Claim 5

An expression vector which comprises the polynucleotide of Claim 3.

Claim 6

An microorganism which is transformed with the

expression vector of Claim 5.

Claim 7

A method for preparing cell-transducing EC SOD fusion protein which comprises the steps of:

(a) transforming a host microorganisms with a recombinant expression vector of Claim 5;

(b) culturing the transformed microorganisms prepared in the step (a) in a suitable medium and condition for the expression of the polynucleotide sequence; and

(c) collecting a substantially pure fusion protein encoded by the polynucleotide sequence from the culture solution of the step (b).

Claim 8

A pharmaceutical composition for preventing or inhibiting ROS related diseases containing fusion protein of Claim 1 or 2 as an effective ingredient.

Claim 9

A pharmaceutical composition for preventing or inhibiting ROS related diseases containing an expression vector of Claim 5.

Claim 10

The pharmaceutical composition of Claim 8 or 9, wherein ROS related diseases are skin diseases.

Claim 11

The pharmaceutical composition of Claim 10, wherein the skin diseases are selected from the group consiting of stin cancer, pigmentation diseases, photo-aging and dermatitis.

Claim 12

A pharmaceutical composition for preventing or inhibiting ROS related skin diseases containing EC SOD protein as an effective ingredient.

Claim 13

The pharmaceutical composition for preventing or inhibiting ROS related skin diseases of Claim 12, wherein the EC SOD protein has the amino acid sequence represented by SEQ ID NO: 11.

Claim 14

A pharmaceutical composition for preventing or inhibiting ROS related skin diseases containing an

expression vector comprising polynucleotides encoding EC SOD protein as an effective ingredient.

Claim 15

The pharmaceutical composition for preventing or inhibiting ROS related skin diseases of Claim 14, wherein the polynucleotides encode EC SOD protein which has amino acid sequence represented SEQ ID NO: 11.

Claim 16

The pharmaceutical composition of Claim 12 or 14, wherein the skin diseases are selected from the group consiting of stin cancer, pigmentation diseases, photo-aging and dermatitis.

Claim 17

A cosmetic composition comprising fusion protien of Claim 12 or 14.

Claim 18

The cosmetic composition of Claim 17, werein the composition is for anti-wrinkle.

Claim 19

A food composition for preventing or inhibiting ROS related diseases comprising fusion protein of Claim 1 or 2.

Claim 20

A food composition for preventing or inhibiting ROS related diseases comprising a microorganism transformed with the expression vector of Claim 5.

Claim 21

A food composition for preventing or inhibiting ROS related skin diseases comprising EC SOD protein.

Claim 22

The food composition for preventing or inhibiting ROS related skin diseases of Claim 21, wherein the EC SOD protein has the amino acid sequence represented by SEQ ID NO: 11.

Claim 23

A food composition for preventing or inhibiting ROS related skin diseases comprising a microorganism transformed with an expression vector comprising polynucleotides encoding EC SOD protein.

Claim 24

The food composition for preventing or inhibiting ROS related skin diseases of Claim 23, wherein the polynucleotides encode EC SOD protein which has amino acid sequence represented SEQ ID NO: 11.

Claim 25

The food composition of Claim 20 or 23, wherein the microorganism is a lactobacillus.

FIG. 1

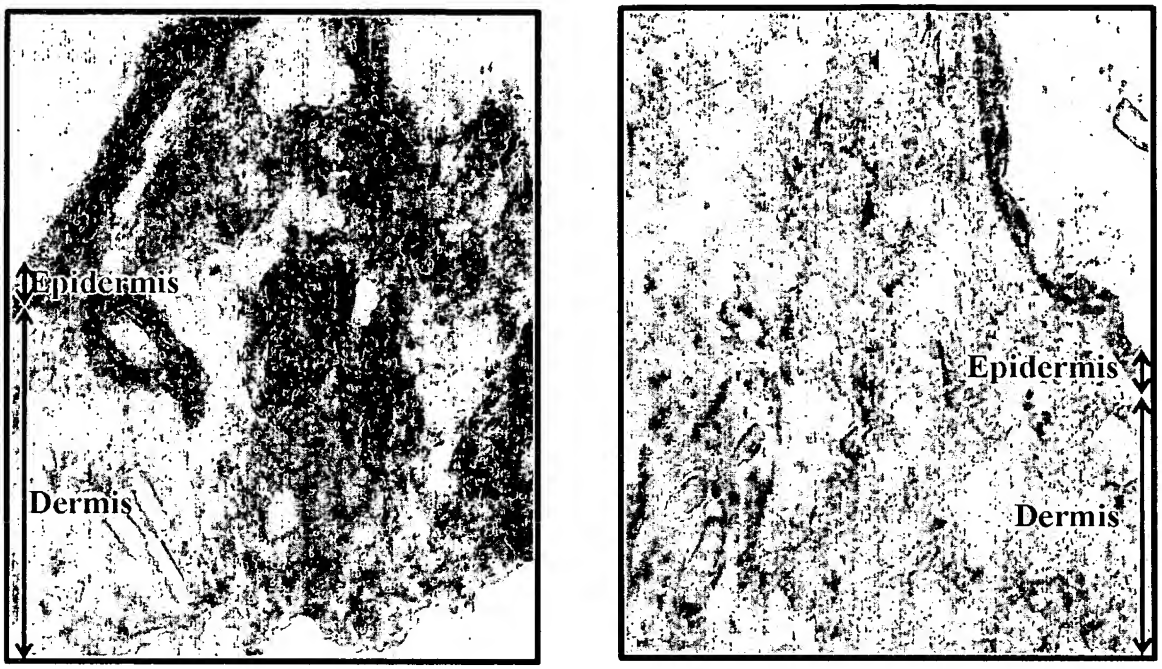


FIG. 2

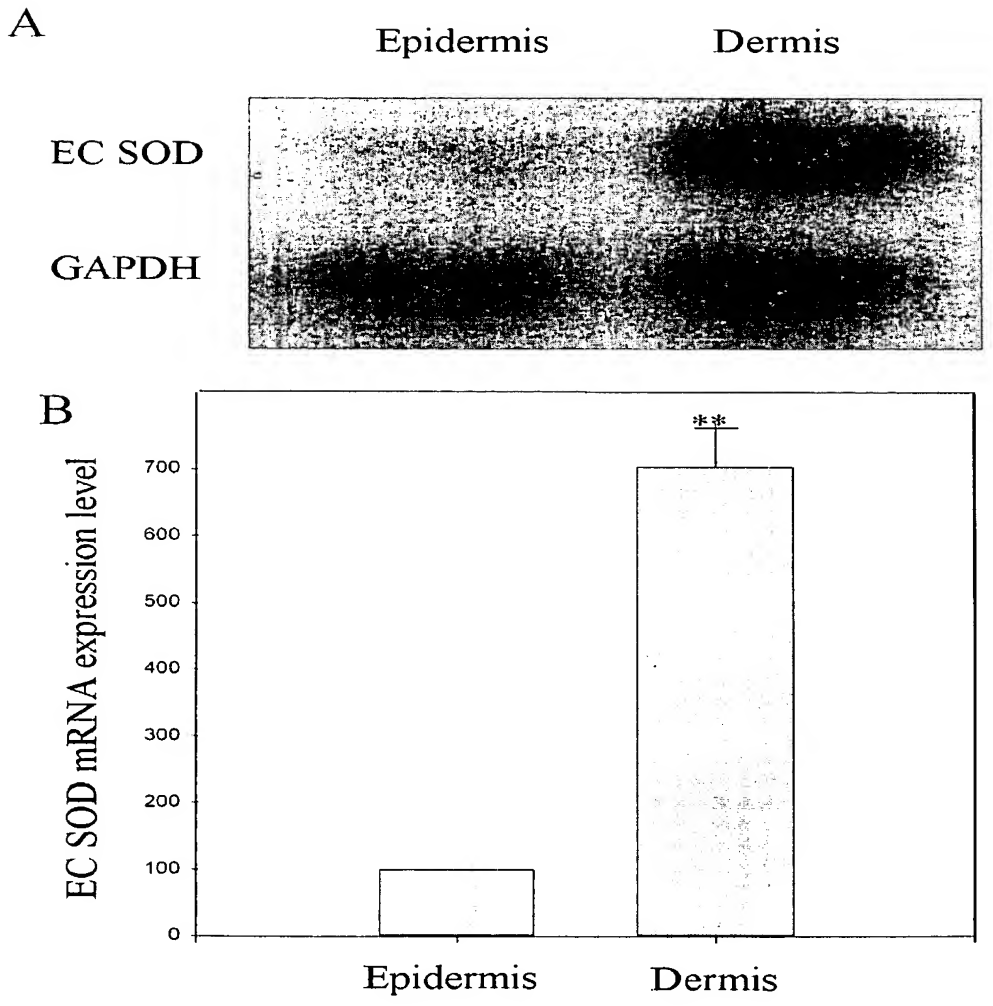

A
Epidermis
Dermis
EC SOD
GAPDH
B
EC SOD mRNA expression level
0
100
200
300
400
500
600
700
**
Epidermis
Dermis

FIG. 3

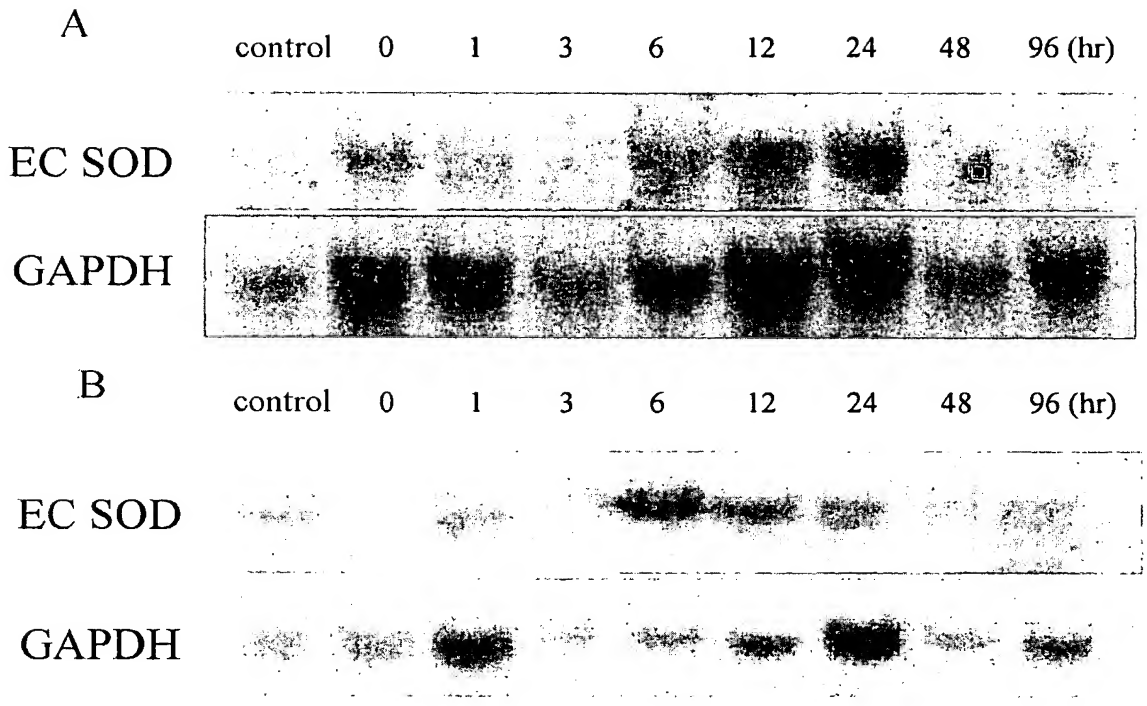

A
control
0
1
3
6
12
24
48
96 (hr)
EC SOD
GAPDH
B
control
0
1
3
6
12
24
48
96 (hr)
EC SOD
GAPDH

FIG. 4

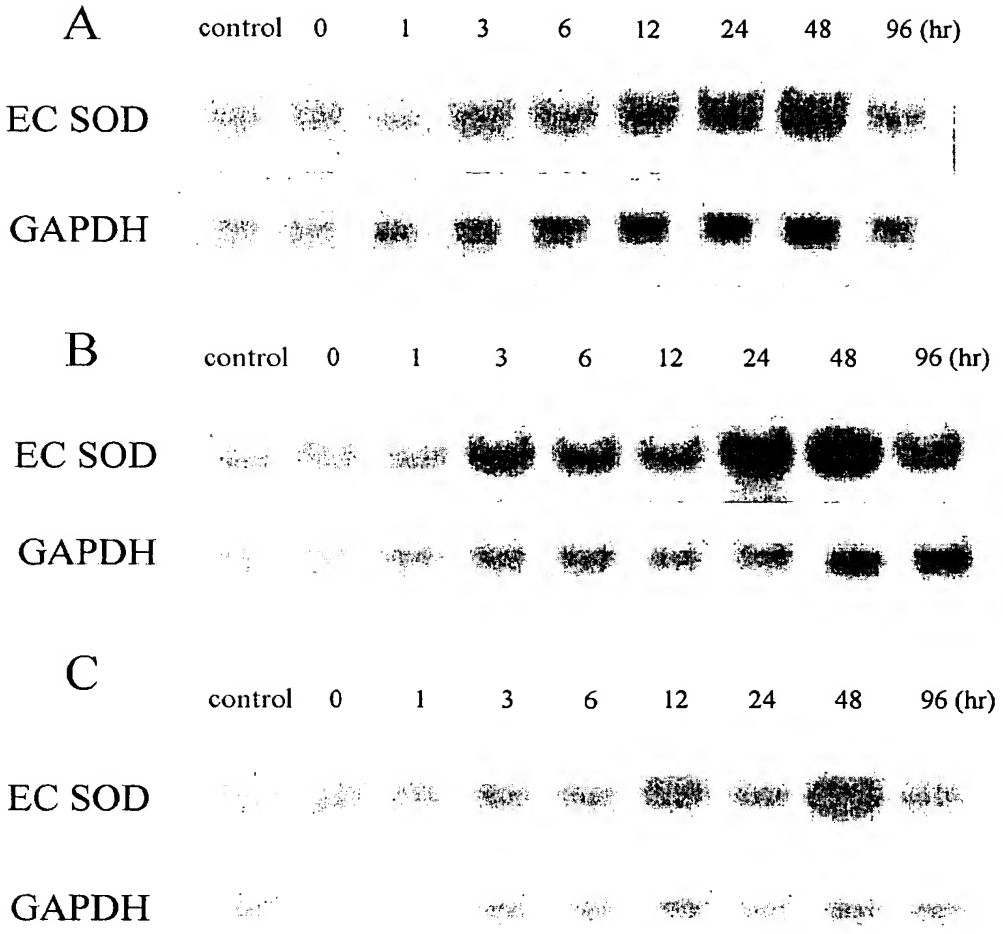

A
control 0 1 3 6 12 24 48 96 (hr)
EC SOD
GAPDH
B
control 0 1 3 6 12 24 48 96 (hr)
EC SOD
GAPDH
C
control 0 1 3 6 12 24 48 96 (hr)
EC SOD
GAPDH

FIG. 5

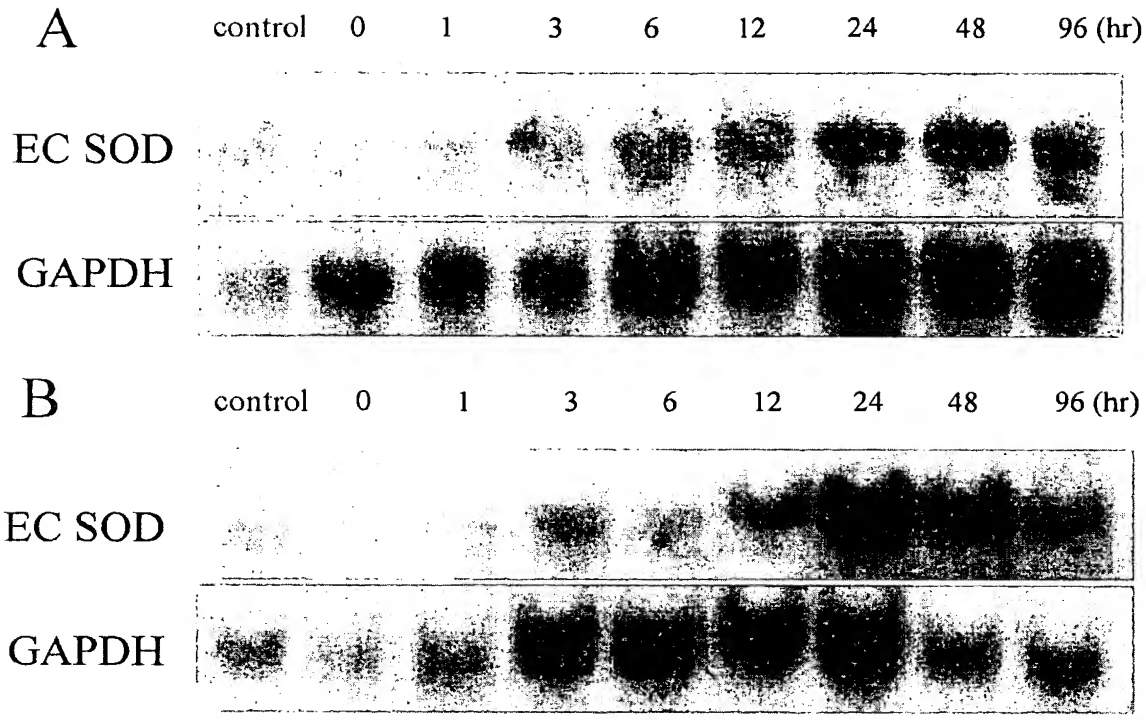

A
control
0
1
3
6
12
24
48
96 (hr)
EC SOD
GAPDH
B
control
0
1
3
6
12
24
48
96 (hr)
EC SOD
GAPDH

FIG. 6

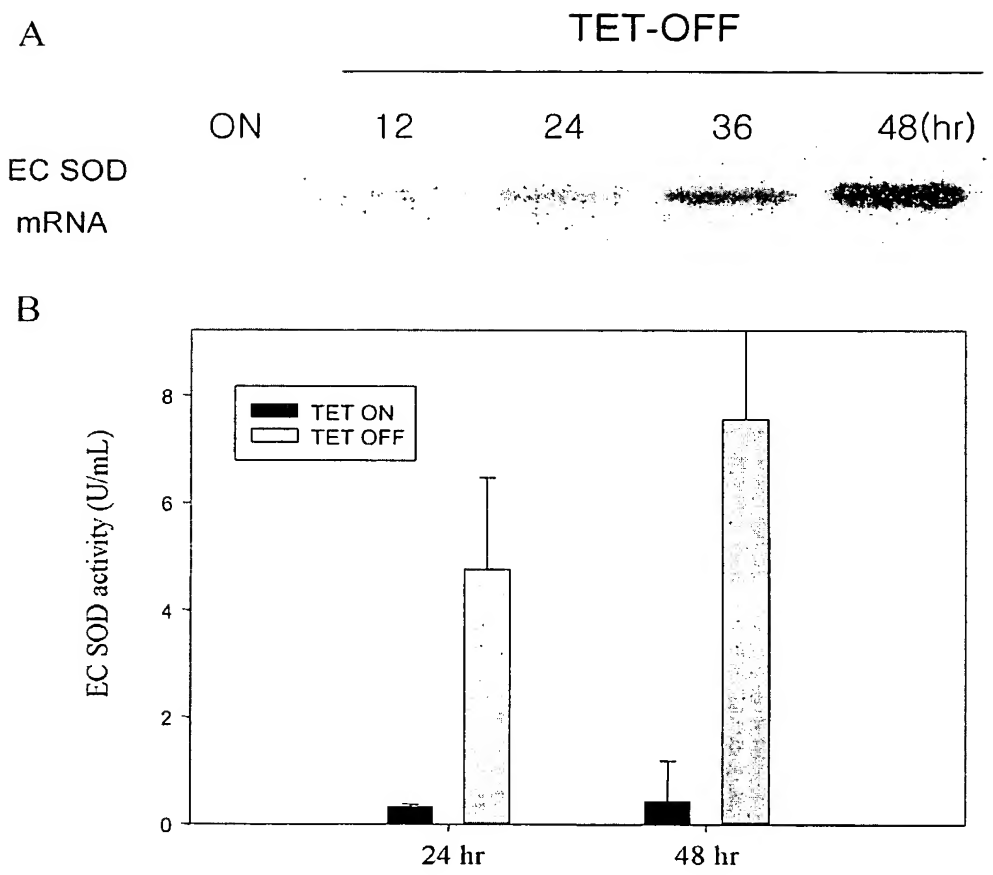

A
TET-OFF
ON
12
24
36
48(hr)
EC SOD
mRNA
B
TET ON
TET OFF
EC SOD activity (U/mL)
0
2
4
6
8
24 hr
48 hr

FIG. 7A

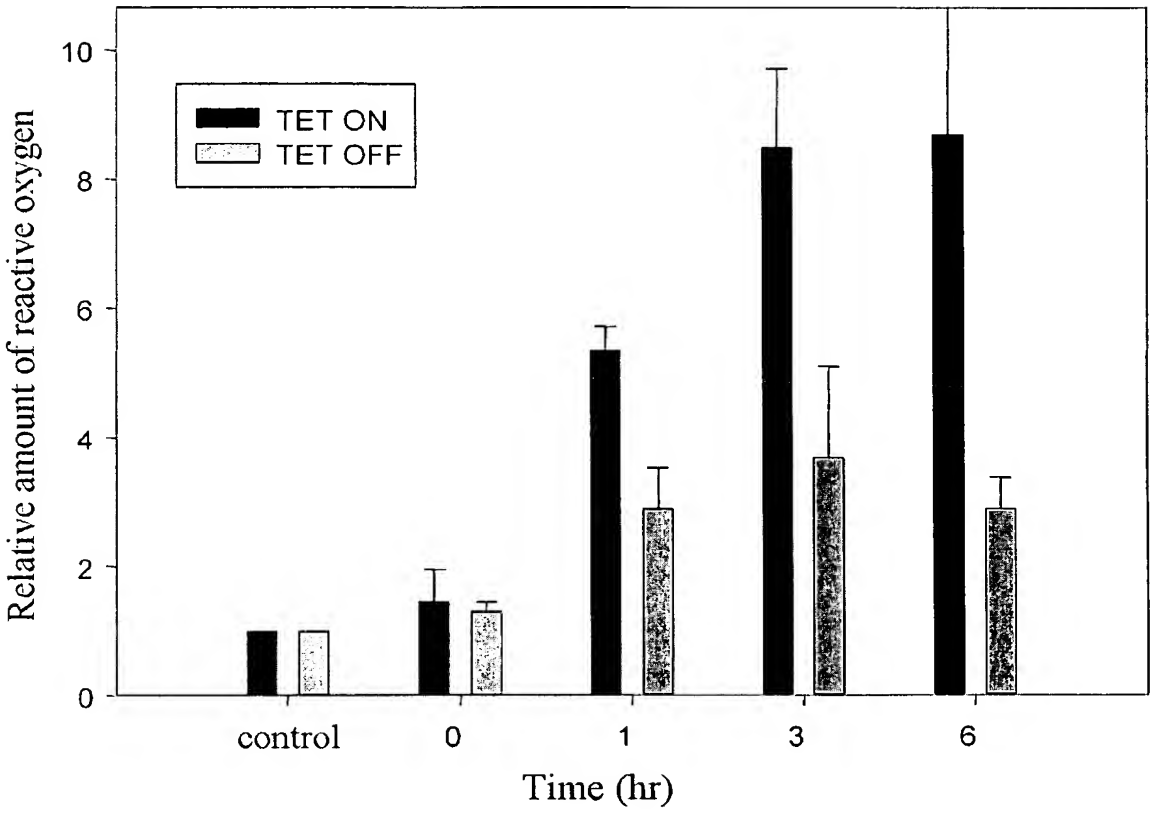

Relative amount of reactive oxygen
10
8
6
4
2
0
TET ON
TET OFF
control
0
1
3
6
Time (hr)

FIG. 7B

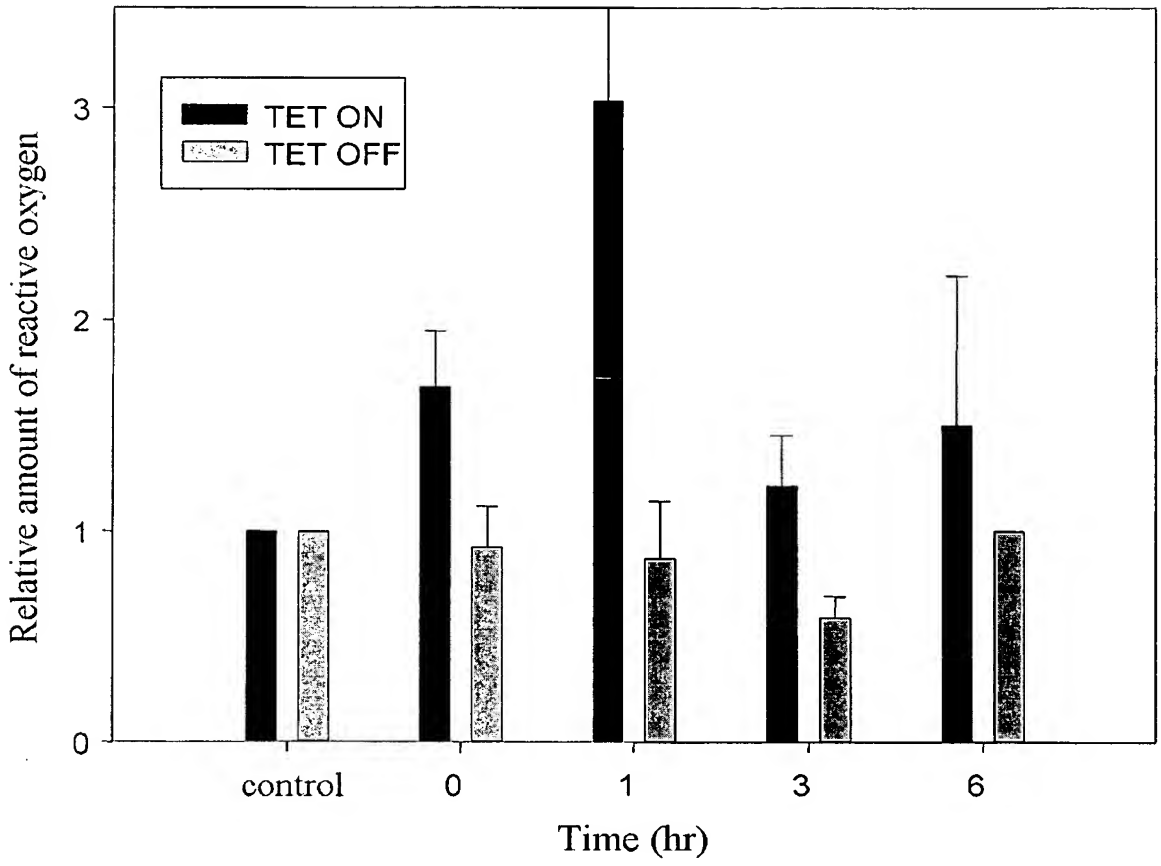

TET ON
TET OFF
Relative amount of reactive oxygen
0
1
2
3
control
0
1
3
6
Time (hr)

FIG. 7C

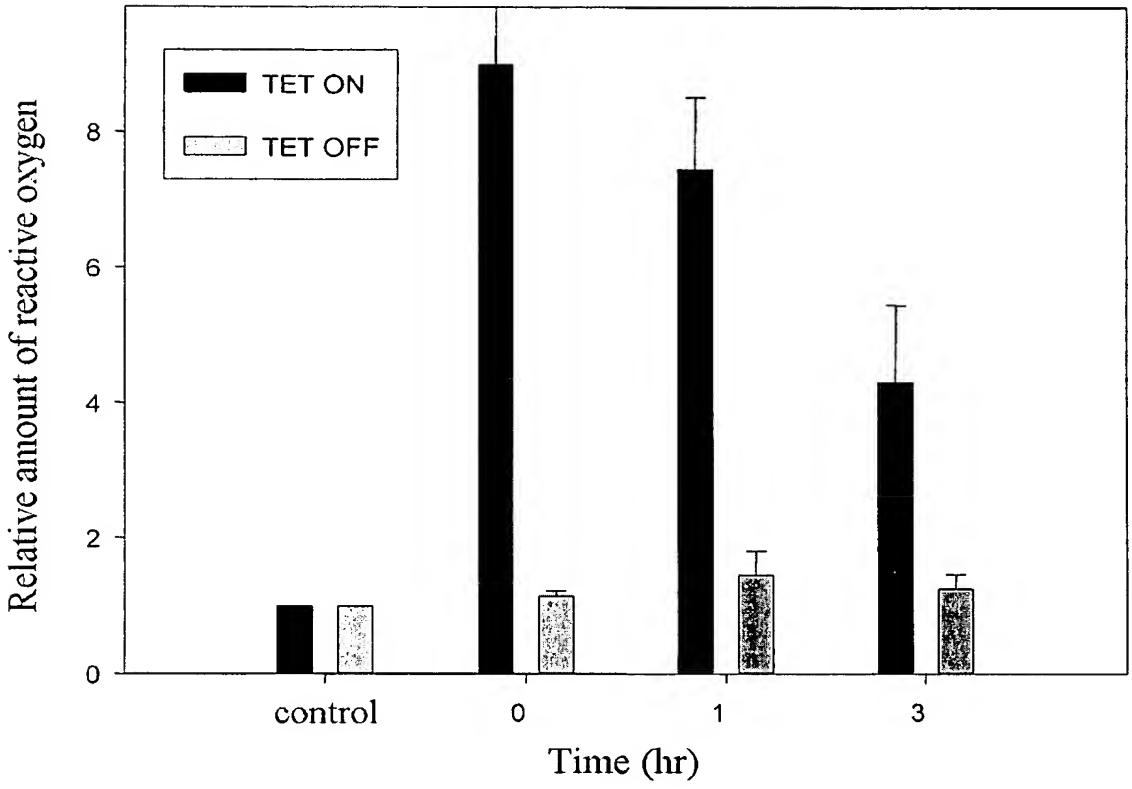

TET ON
TET OFF
Relative amount of reactive oxygen
0
2
4
6
8
control
0
1
3
Time (hr)

FIG. 8

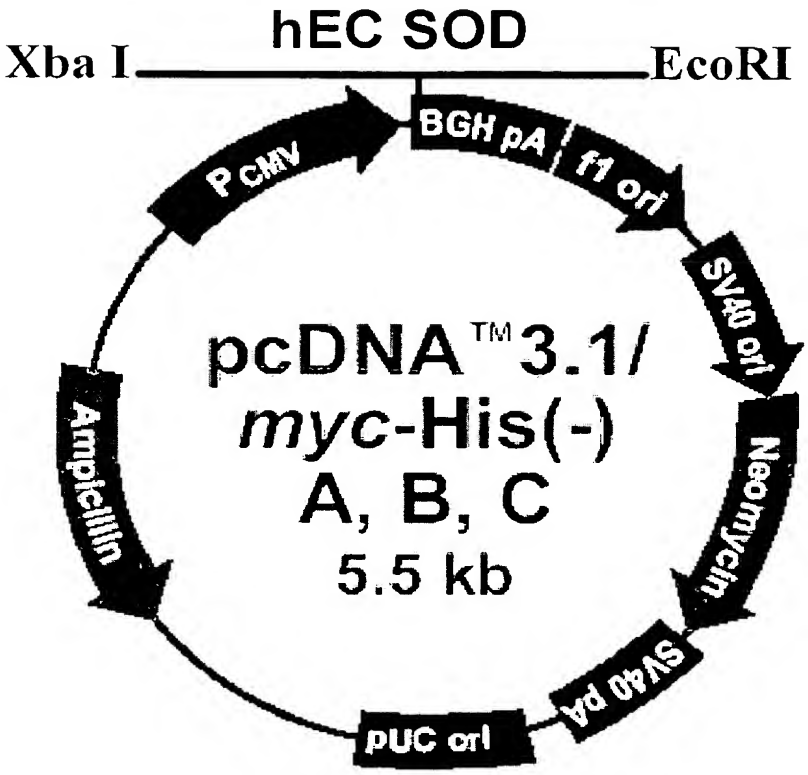

hEC SOD
Xba I
EcoRI
P CMV
BGH pA
f1 ori
SV40 ori
Neomycin
SV40 pA
pUC ori
Ampicillin
pcDNA™3.1/
myc-His(-)
A, B, C
5.5 kb

FIG. 9

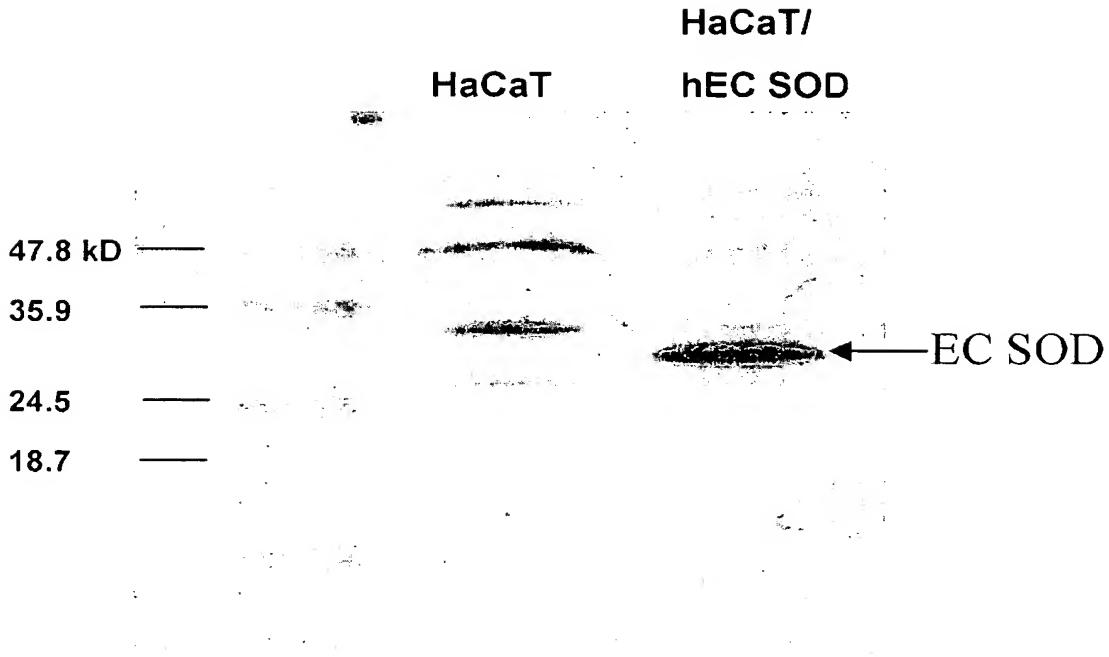


FIG. 10

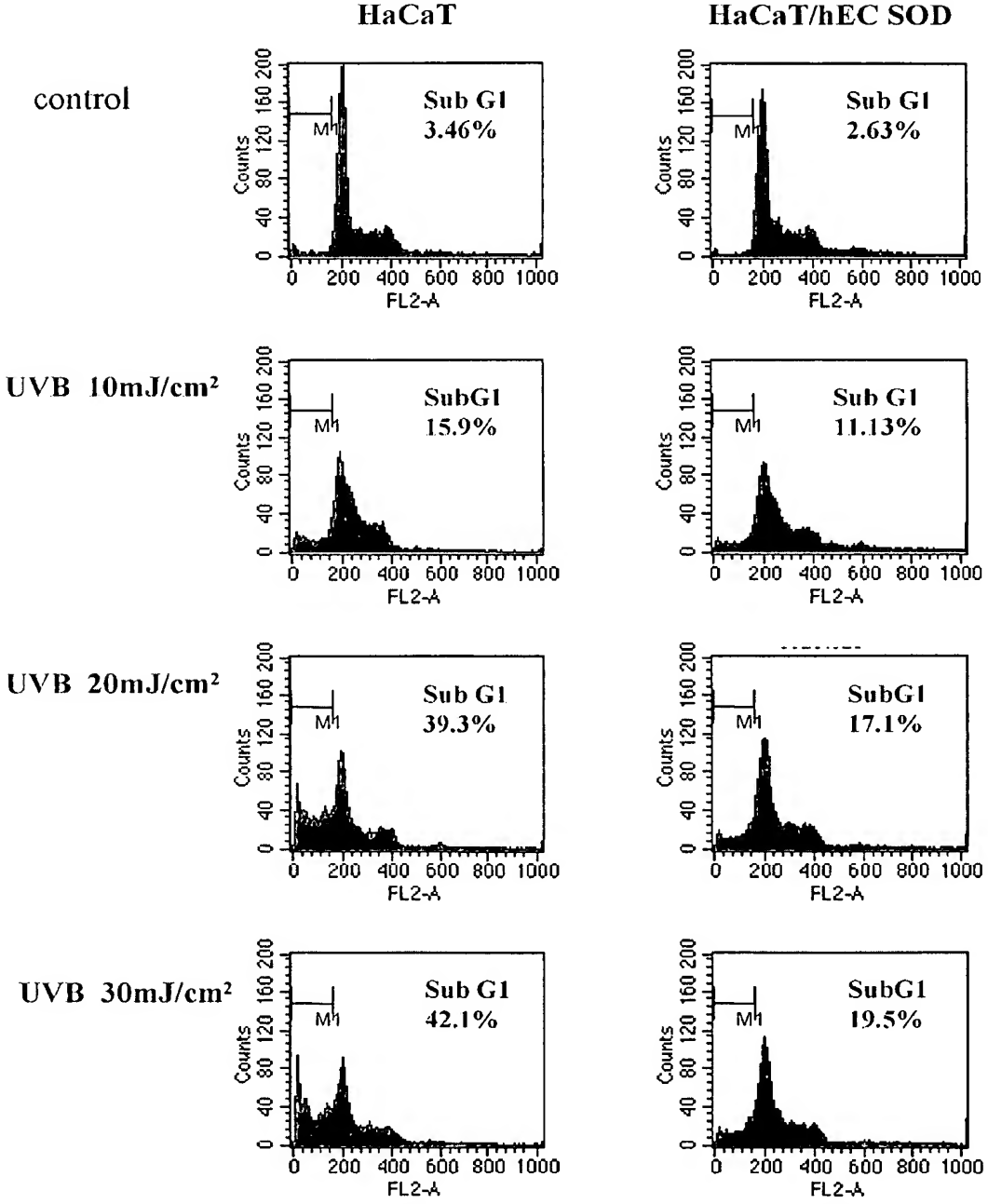

HaCaT
HaCaT/hEC SOD
control
UVB 10mJ/cm²
UVB 20mJ/cm²
UVB 30mJ/cm²
Sub G1
3.46%
Sub G1
2.63%
SubG1
15.9%
Sub G1
11.13%
Sub G1
39.3%
SubG1
17.1%
Sub G1
42.1%
SubG1
19.5%
M1
Counts
FL2-A
0 40 80 120 160 200
0 200 400 600 800 1000

FIG. 11

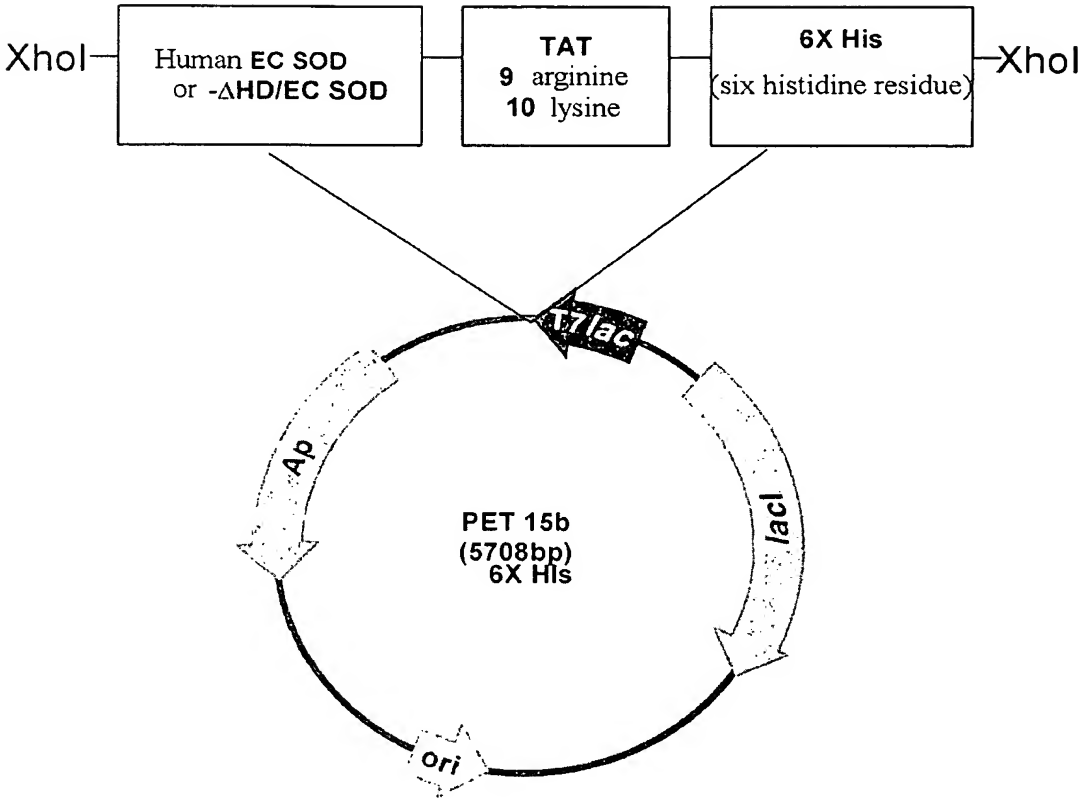

XhoI
Human EC SOD
or -ΔHD/EC SOD
TAT
9 arginine
10 lysine
6X His
(six histidine residue)
XhoI
T7lac
Ap
PET 15b
(5708bp)
6X His
lacI
ori

FIG. 12

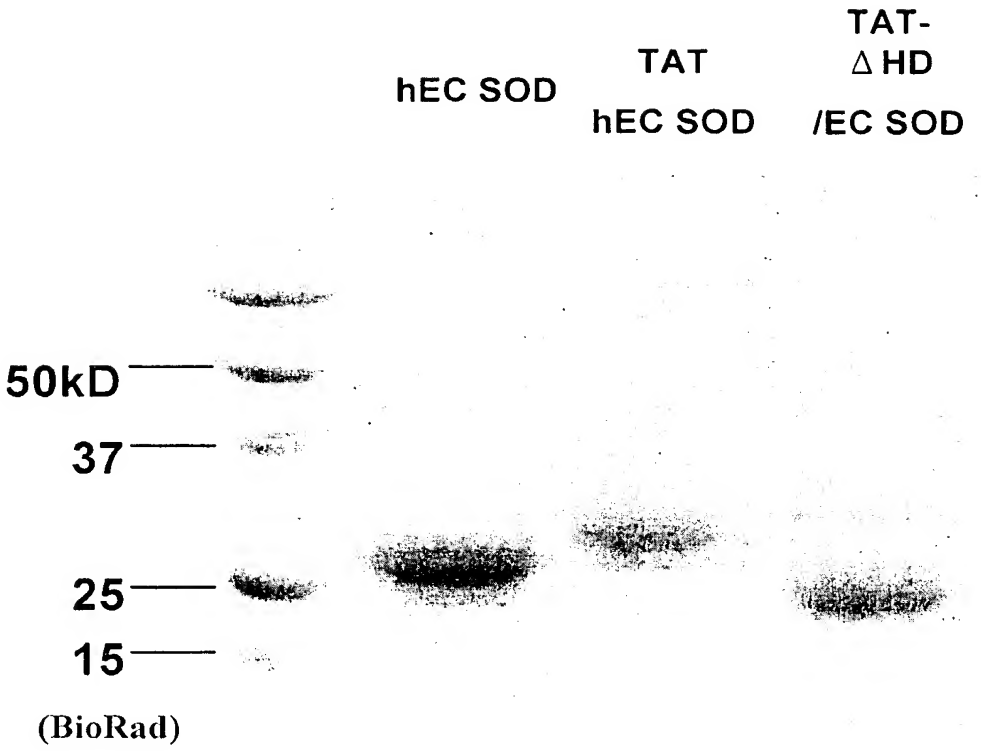


FIG. 13

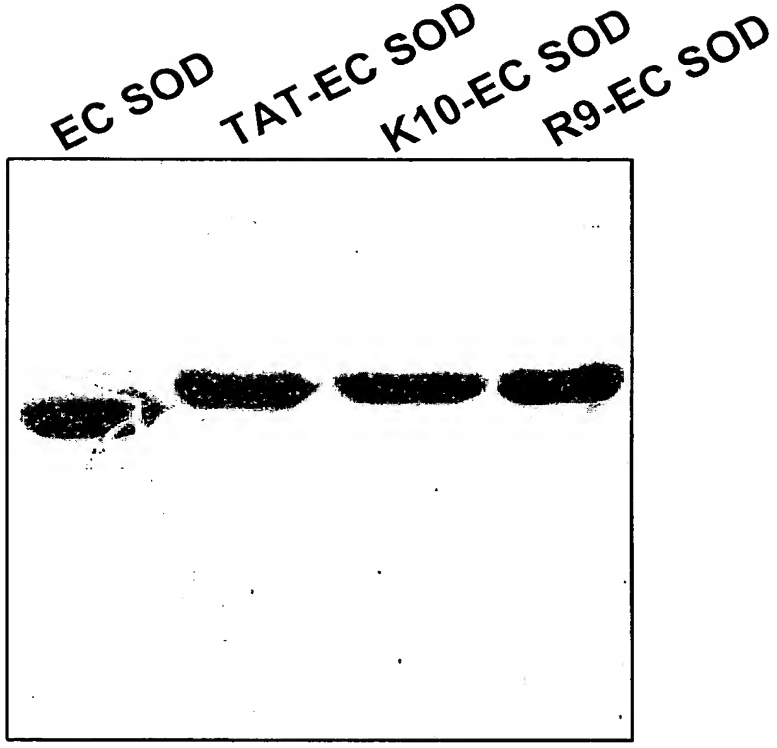


FIG. 14

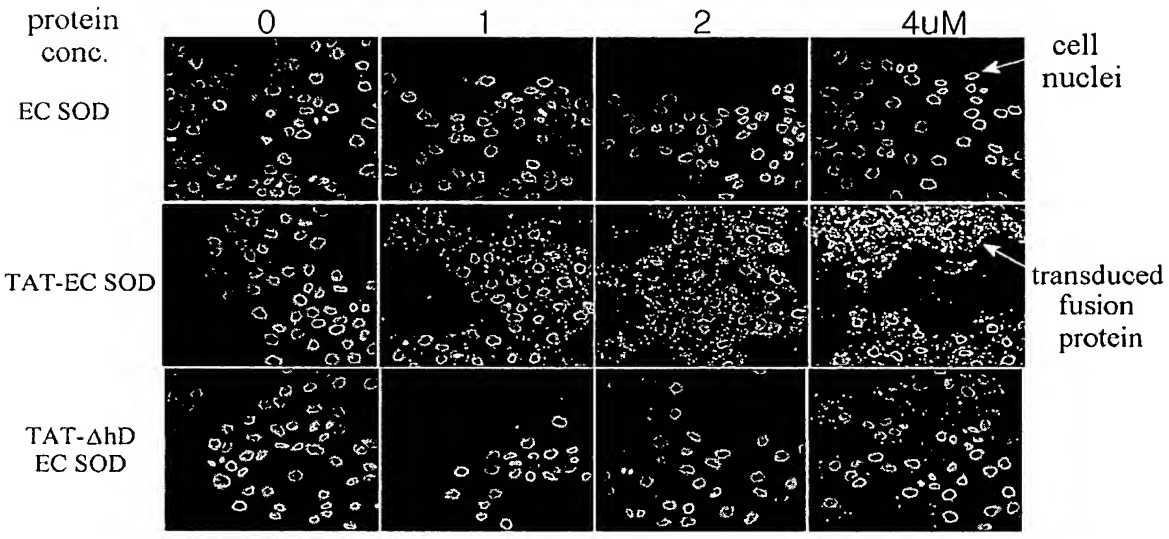
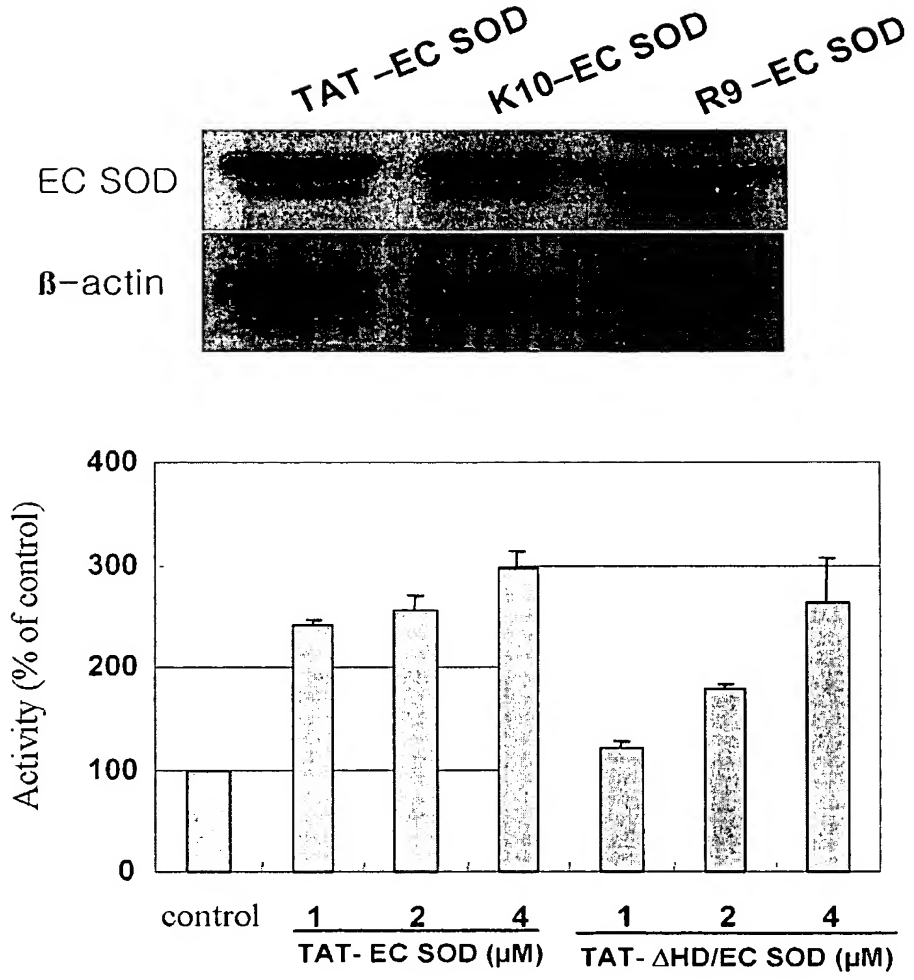

protein conc.
0
1
2
4uM
EC SOD
TAT-EC SOD
TAT-ΔhD EC SOD
cell nuclei
transduced fusion protein

FIG. 15


TAT -EC SOD
K10-EC SOD
R9 -EC SOD
EC SOD
ß-actin
Activity (% of control)
400
300
200
100
0
control
1
2
4
TAT- EC SOD (μM)
1
2
4
TAT- ΔHD/EC SOD (μM)

SEQUENCE LISTING

<110> KIM, TAE-YOON

BIO CLUE & SOLUTION CO., LT

<120> EC SOD and Cell transducing EC SOD and use thereof

<150> KR10-2003-0076629

<151> 2003-10-31

<160> 33

<170> KopatentIn 1.71

<210> 1

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 1

atgttggcct tcttgttc 18

<210> 2

<211> 18

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 2

ttaagtggtc ttgcactc 18

<210> 3

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 3

agtctcgaga tgttggcctt cttgttctac ggc 33

<210> 4

<211> 28

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 4

gatcctcgag tggtcttgca ctcgctct 28

<210> 5

<211> 27

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 5

atctctagaa tgctggcgct actgtgt 27

<210> 6

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 6

atcgaatcct caggcggcct tgcactcgct ctct 34

<210> 7

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 7

gatcctcgag tggacgggcg aggactcggc 30

<210> 8

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 8

gatcctcgag tcaggcggcc ttgcactcgc t 31

<210> 9

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 9

gatcctcgag tggacgggcg aggactcggc 30

<210> 10

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> primer

<400> 10

aatgctcgag tcactctgag tgctcccgcg c 31

<210> 11

<211> 240

<212> PRT

<213> Homo sapiens

<220>

<221> PEPTIDE

<222> (1)..(240)

<223> Human EC SOD

<400> 11

Met Leu Ala Leu Leu Cys Ser Cys Leu Leu Leu Ala Ala Gly Ala Ser
1 5 10 15

Asp Ala Trp Thr Gly Glu Asp Ser Ala Glu Pro Asn Ser Asp Ser Ala
20 25 30

Glu Trp Ile Arg Asp Met Tyr Ala Lys Val Thr Glu Ile Trp Gln Glu
35 40 45

Val Met Gln Arg Arg Asp Asp Asp Gly Thr Leu His Ala Ala Cys Gln
50 55 60

Val Gln Pro Ser Ala Thr Leu Asp Ala Ala Gln Pro Arg Val Thr Gly
65 70 75 80

Val Val Leu Phe Arg Gln Leu Ala Pro Arg Ala Lys Leu Asp Ala Phe

85 90 95

Phe Ala Leu Glu Gly Phe Pro Thr Glu Pro Asn Ser Ser Ser Arg Ala

100 105 110

Ile His Val His Gln Phe Gly Asp Leu Ser Gln Gly Cys Glu Ser Thr

115 120 125

Gly Pro His Tyr Asn Pro Leu Ala Val Pro His Pro Gln His Pro Gly

130 135 140

Asp Phe Gly Asn Phe Ala Val Arg Asp Gly Ser Leu Trp Arg Tyr Arg

145 150 155 160

Ala Gly Leu Ala Ala Ser Leu Ala Gly Pro His Ser Ile Val Gly Arg

165 170 175

Ala Val Val Val His Ala Gly Glu Asp Asp Leu Gly Arg Gly Gly Asn

180 185 190

Gln Ala Ser Val Glu Asn Gly Asn Ala Gly Arg Arg Leu Ala Cys Cys

195 200 205

Val Val Gly Val Cys Gly Pro Gly Leu Trp Glu Arg Gln Ala Arg Glu

210 215 220

His Ser Glu Arg Lys Lys Arg Arg Arg Glu Ser Glu Cys Lys Ala Ala

225 230 235 240

<210> 12

<211> 231

<212> PRT

<213> Artificial Sequence

<220>

<223> TAT-EC SOD fusion protein

<400> 12

Arg Lys Lys Arg Arg Gln Arg Arg Arg Trp Thr Gly Glu Asp Ser Ala
1 5 10 15

Glu Pro Asn Ser Asp Ser Ala Glu Trp Ile Arg Asp Met Tyr Ala Lys
20 25 30

Val Thr Glu Ile Trp Gln Glu Val Met Gln Arg Arg Asp Asp Asp Gly
35 40 45

Thr Leu His Ala Ala Cys Gln Val Gln Pro Ser Ala Thr Leu Asp Ala
50 55 60

Ala Gln Pro Arg Val Thr Gly Val Val Leu Phe Arg Gln Leu Ala Pro
65 70 75 80

Arg Ala Lys Leu Asp Ala Phe Phe Ala Leu Glu Gly Phe Pro Thr Glu
85 90 95

Pro Asn Ser Ser Ser Arg Ala Ile His Val His Gln Phe Gly Asp Leu
100 105 110

Ser Gln Gly Cys Glu Ser Thr Gly Pro His Tyr Asn Pro Leu Ala Val
115 120 125

Pro His Pro Gln His Pro Gly Asp Phe Gly Asn Phe Ala Val Arg Asp

130 135 140

Gly Ser Leu Trp Arg Tyr Arg Ala Gly Leu Ala Ala Ser Leu Ala Gly

145 150 155 160

Pro His Ser Ile Val Gly Arg Ala Val Val Val His Ala Gly Glu Asp

165 170 175

Asp Leu Gly Arg Gly Gly Asn Gln Ala Ser Val Glu Asn Gly Asn Ala

180 185 190

Gly Arg Arg Leu Ala Cys Cys Val Val Gly Val Cys Gly Pro Gly Leu

195 200 205

Trp Glu Arg Gln Ala Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg

210 215 220

Glu Ser Glu Cys Lys Ala Ala

225 230

<210> 13

<211> 218

<212> PRT

<213> Artificial Sequence

<220>

<223> TAT-delta HD/EC SOD fusion protein

<400> 13

Arg Lys Lys Arg Arg Gln Arg Arg Arg Trp Thr Gly Glu Asp Ser Ala

1 5 10 15

Glu Pro Asn Ser Asp Ser Ala Glu Trp Ile Arg Asp Met Tyr Ala Lys

20 25 30

Val Thr Glu Ile Trp Gln Glu Val Met Gln Arg Arg Asp Asp Asp Gly

35 40 45

Thr Leu His Ala Ala Cys Gln Val Gln Pro Ser Ala Thr Leu Asp Ala

50 55 60

Ala Gln Pro Arg Val Thr Gly Val Val Leu Phe Arg Gln Leu Ala Pro

65 70 75 80

Arg Ala Lys Leu Asp Ala Phe Phe Ala Leu Glu Gly Phe Pro Thr Glu

85 90 95

Pro Asn Ser Ser Ser Arg Ala Ile His Val His Gln Phe Gly Asp Leu

100 105 110

Ser Gln Gly Cys Glu Ser Thr Gly Pro His Tyr Asn Pro Leu Ala Val

115 120 125

Pro His Pro Gln His Pro Gly Asp Phe Gly Asn Phe Ala Val Arg Asp

130 135 140

Gly Ser Leu Trp Arg Tyr Arg Ala Gly Leu Ala Ala Ser Leu Ala Gly

145 150 155 160

Pro His Ser Ile Val Gly Arg Ala Val Val Val His Ala Gly Glu Asp

165 170 175

Asp Leu Gly Arg Gly Gly Asn Gln Ala Ser Val Glu Asn Gly Asn Ala

180 185 190

Gly Arg Arg Leu Ala Cys Cys Val Val Gly Val Cys Gly Pro Gly Leu

195 200 205

Trp Glu Arg Gln Ala Arg Glu His Ser Glu

210 215

<210> 14

<211> 231

<212> PRT

<213> Artificial Sequence

<220>

<223> R9-EC SOD fusion protein

<400> 14

Arg Arg Arg Arg Arg Arg Arg Arg Arg Trp Thr Gly Glu Asp Ser Ala

1 5 10 15

Glu Pro Asn Ser Asp Ser Ala Glu Trp Ile Arg Asp Met Tyr Ala Lys

20 25 30

Val Thr Glu Ile Trp Gln Glu Val Met Gln Arg Arg Asp Asp Asp Gly

35 40 45

Thr Leu His Ala Ala Cys Gln Val Gln Pro Ser Ala Thr Leu Asp Ala

50 55 60

Ala Gln Pro Arg Val Thr Gly Val Val Leu Phe Arg Gln Leu Ala Pro

65 70 75 80

Arg Ala Lys Leu Asp Ala Phe Phe Ala Leu Glu Gly Phe Pro Thr Glu

85 90 95

Pro Asn Ser Ser Ser Arg Ala Ile His Val His Gln Phe Gly Asp Leu

100 105 110

Ser Gln Gly Cys Glu Ser Thr Gly Pro His Tyr Asn Pro Leu Ala Val

115 120 125

Pro His Pro Gln His Pro Gly Asp Phe Gly Asn Phe Ala Val Arg Asp

130 135 140

Gly Ser Leu Trp Arg Tyr Arg Ala Gly Leu Ala Ala Ser Leu Ala Gly

145 150 155 160

Pro His Ser Ile Val Gly Arg Ala Val Val Val His Ala Gly Glu Asp

165 170 175

Asp Leu Gly Arg Gly Gly Asn Gln Ala Ser Val Glu Asn Gly Asn Ala

180 185 190

Gly Arg Arg Leu Ala Cys Cys Val Val Gly Val Cys Gly Pro Gly Leu

195 200 205

Trp Glu Arg Gln Ala Arg Glu His Ser Glu Arg Lys Lys Arg Arg Arg

210 215 220

Glu Ser Glu Cys Lys Ala Ala

225 230

<210> 15

<211> 232

<212> PRT

<213> Artificial Sequence

<220>

<223> K10-EC SOD fusion protein

<400> 15

Lys Lys Lys Lys Lys Lys Lys Lys Lys Lys Trp Thr Gly Glu Asp Ser

1 5 10 15

Ala Glu Pro Asn Ser Asp Ser Ala Glu Trp Ile Arg Asp Met Tyr Ala

20 25 30

Lys Val Thr Glu Ile Trp Gln Glu Val Met Gln Arg Arg Asp Asp Asp

35 40 45

Gly Thr Leu His Ala Ala Cys Gln Val Gln Pro Ser Ala Thr Leu Asp

50 55 60

Ala Ala Gln Pro Arg Val Thr Gly Val Val Leu Phe Arg Gln Leu Ala

65 70 75 80

Pro Arg Ala Lys Leu Asp Ala Phe Phe Ala Leu Glu Gly Phe Pro Thr

85 90 95

Glu Pro Asn Ser Ser Ser Arg Ala Ile His Val His Gln Phe Gly Asp

100 105 110

Leu Ser Gln Gly Cys Glu Ser Thr Gly Pro His Tyr Asn Pro Leu Ala

115 120 125

Val Pro His Pro Gln His Pro Gly Asp Phe Gly Asn Phe Ala Val Arg

130 135 140

Asp Gly Ser Leu Trp Arg Tyr Arg Ala Gly Leu Ala Ala Ser Leu Ala

145 150 155 160

Gly Pro His Ser Ile Val Gly Arg Ala Val Val Val His Ala Gly Glu

165 170 175

Asp Asp Leu Gly Arg Gly Gly Asn Gln Ala Ser Val Glu Asn Gly Asn

180 185 190

Ala Gly Arg Arg Leu Ala Cys Cys Val Val Gly Val Cys Gly Pro Gly

195 200 205

Leu Trp Glu Arg Gln Ala Arg Glu His Ser Glu Arg Lys Lys Arg Arg

210 215 220

Arg Glu Ser Glu Cys Lys Ala Ala

225 230

<210> 16

<211> 696

<212> DNA

<213> Artificial Sequence

<220>

<223> nucloetide sequence encoding TAT-EC SOD fusion protein

<400> 16

aggaagaagc ggagacagcg acgaagatgg acgggcgagg actcggcgga gcccaactct 60

gactcggcgg agtggatccg agacatgtac gccaaggtca cggagatctg gcaggaggtc 120

atgcagcggc gggacgacga cggcacgctc cacgccgcct gccaggtgca gccgtcggcc 180

acgctggacg ccgcgcagcc ccgggtgacc ggcgtcgtcc tcttccggca gcttgcgccc 240

cgcgccaagc tcgacgcctt cttcgccctg gagggcttcc cgaccgagcc gaacagctcc 300

agccgcgcca tccacgtgca ccagttcggg gacctgagcc agggctgcga gtccaccggg 360

ccccactaca acccgctggc cgtgccgcac ccgcagcacc cgggcgactt cggcaacttc 420

gcggtccgcg acggcagcct ctggaggtac cgcgccggcc tggccgcctc gctcgcgggc 480

ccgcactcca tcgtgggccg ggccgtggtc gtccacgctg gcgaggacga cctgggccgc 540

ggcggcaacc aggccagcgt ggagaacggg aacgcgggcc ggcggctggc ctgctgcgtg 600

gtgggcgtgt gcgggcccgg gctctgggag cgccaggcgc gggagcactc agagcgcaag 660

aagcggcggc gcgagagcga gtgcaaggcc gcctga 696

<210> 17

<211> 657

<212> DNA

<213> Artificial Sequence

<220>

<223> nucleotide sequence encoding TAT-delta HD/EC SOD fusion protein

<400> 17

aggaagaagc ggagacagcg acgaagatgg acgggcgagg actcggcgga gcccaactct 60

gactcggcgg agtggatccg agacatgtac gccaaggtca cggagatctg gcaggaggtc 120

atgcagcggc gggacgacga cggcacgctc cacgccgcct gccaggtgca gccgtcggcc 180

acgctggacg ccgcgcagcc ccgggtgacc ggcgtcgtcc tcttccggca gcttgcgccc 240

cgcgccaagc tcgacgcctt cttcgccctg gagggcttcc cgaccgagcc gaacagctcc 300

agccgcgcca tccacgtgca ccagttcggg gacctgagcc agggctgcga gtccaccggg 360

ccccactaca acccgctggc cgtgccgcac ccgcagcacc cgggcgactt cggcaacttc 420

gcggtccgcg acggcagcct ctggaggtac cgcgccggcc tggccgcctc gctcgcgggc 480

ccgcactcca tcgtgggccg ggccgtggtc gtccacgctg gcgaggacga cctgggccgc 540

ggcggcaacc aggccagcgt ggagaacggg aacgcgggcc ggcggctggc ctgctgcgtg 600

gtgggcgtgt gcgggcccgg gctctgggag cgccaggcgc gggagcactc agagtga 657

<210> 18

<211> 696

<212> DNA

<213> Artificial Sequence

<220>

<223> nucleotide sequence encoding R9-EC SOD fusion protein

<400> 18

cggcggcggc ggcggcggcg gcggcggtgg acgggcgagg actcggcgga gcccaactct 60

gactcggcgg agtggatccg agacatgtac gccaaggtca cggagatctg gcaggaggtc 120

atgcagcggc gggacgacga cggcacgctc cacgccgcct gccaggtgca gccgtcggcc 180

acgctggacg ccgcgcagcc ccgggtgacc ggcgtcgtcc tcttccggca gcttgcgccc 240

cgcgccaagc tcgacgcctt cttcgccctg gagggcttcc cgaccgagcc gaacagctcc 300

agccgcgcca tccacgtgca ccagttcggg gacctgagcc agggctgcga gtccaccggg 360

ccccactaca acccgctggc cgtgccgcac ccgcagcacc cgggcgactt cggcaacttc 420

gcggtccgcg acggcagcct ctggaggtac cgcgccggcc tggccgcctc gctcgcgggc 480

ccgcactcca tcgtgggccg ggccgtggtc gtccacgctg gcgaggacga cctgggccgc 540

ggcggcaacc aggccagcgt ggagaacggg aacgcgggcc ggcggctggc ctgctgcgtg 600

gtgggcgtgt gcgggcccgg gctctgggag cgccaggcgc gggagcactc agagcgcaag 660

aagcggcggc gcgagagcga gtgcaaggcc gcctga 696

<210> 19

<211> 699

<212> DNA

<213> Artificial Sequence

<220>

<223> nucleotide sequence encoding R9-EC SOD fusion protein

<400> 19

aagaagaaga agaagaagaa gaagaagaag tggacgggcg aggactcggc ggagcccaac 60

tctgactcgg cggagtggat ccgagacatg tacgccaagg tcacggagat ctggcaggag 120

gtcatgcagc ggcgggacga cgacggcacg ctccacgccg cctgccaggt gcagccgtcg 180

gccacgctgg acgccgcgca gccccgggtg accggcgtcg tcctcttccg gcagcttgcg 240

ccccgcgcca agctcgacgc cttcttcgcc ctggagggct tcccgaccga gccgaacagc 300

tccagccgcg ccatccacgt gcaccagttc ggggacctga gccagggctg cgagtccacc 360

gggccccact acaacccgct ggccgtgccg cacccgcagc acccgggcga cttcggcaac 420

ttcgcggtcc gcgacggcag cctctggagg taccgcgccg gcctggccgc ctcgctcgcg 480

ggcccgcact ccatcgtggg ccgggccgtg gtcgtccacg ctggcgagga cgacctgggc 540

cgcggcggca accaggccag cgtggagaac gggaacgcgg gccggcggct ggcctgctgc 600

gtggtgggcg tgtgcgggcc cgggctctgg gagcgccagg cgcgggagca ctcagagcgc 660

aagaagcggc ggcgcgagag cgagtgcaag gccgcctga 699